NELSON MANDELA UNIVERSITY

FINDING FRANKENFLORA: INVESTIGATING HYBRIDISATION BETWEEN LOCAL AND INTRODUCED *PROTEA* SPECIES AT THE VAN STADENS WILDFLOWER RESERVE

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Finding Frankenflora: Investigating hybridisation between local and introduced *Protea* species at the Van Stadens Wildflower Reserve

By

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Contents

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Abstract

Aims. This study provides an overview of hybridisation and methods to detect it in plants. This is documented in chapter 1. Another aim was to identify hybridisation between local and non-local species in the Proteaceae. This is covered in chapter 2. Finally the third aim was to develop a cheap and rapidly working method of detecting hybrids in this system. This is detailed in chapter 3.

Location. The model system for this research is the Van Stadens Wildflower Reserve. In 1984 the reserve manager introduced the non-local species, *Protea susannae* from Cape Agulhas. This represents a long-distance dispersal of over 600 km. This study is focused on looking at the impacts of the introduction of *P. susannae* on the local species *P. eximia*.

Methods. I use genetic and morphological methods to detect hybrids. Hybridisation and the methods implemented to identify it are reviewed and discussed (Chapter 1). Morphological traits of the two species and direct sequencing of the nuclear ITS and chloroplast regions are compared (Chapter 2). This includes notes on the detection of hybrids and the potential for cryptic hybrids. I also focussed on testing of two rapid and low-cost techniques for detecting hybrids using Species Specific Primers (SSP's) and High Resolution Melt (HRM).

Results. The Sanger sequencing method and ITS detected cryptic hybrids. The HRM was able to detect hybrids when comparing F_1 putative hybrids to a 'pure' parent populations, but was not able to detect between a 'pure' parent population and F_3 or F_4 putative hybrids in the Van Stadens system. The SSP's had the highest hybrid detection rate. Genetic methods, specifically the SSP's were found to work well to detect hybrids in an environment where a hybrid swarm has taken place. The maps produced from this thesis will be useful for determining the distribution of hybrids in the reserve and for other similar hybrid systems.

Conclusions. Long distance dispersal of Cape species may lead to the loss of genetic diversity or species as local and non-local hybridisation may occur, similar to the hybridisation between *P. susannae* and *P. eximia* in the Van Stadens Wild Flower Reserve. Genetic methods were shown to be superior to morphological hybrid detection (i.e. Sanger sequencing and SSP's). The SSP approach tested here, which is dependent on the presence of fixed species specific SNP's (Single Nucleotide Polymorphisms), could offer a rapid and effective method to explore hybridisation through space and time.

Acknowledgements

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DECLARATION BY CANDIDATE

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STUDENT NUMBER: 21225 3336

QUALIFICATION: Master of Science (Msc)

TITLE OF PROJECT: Finding Frankenflora: Investigating hybridisation between local and introduced Protea species at the Van Stadens Wildflower Reserve

DECLARATION:

In accordance with Rule G5.6.3, I hereby declare that the above-mentioned treatise/ dissertation/ thesis is my own work and that it has not previously been submitted for assessment to another University or for another qualification.

SIGNATURE: T. P Macqueen

DATE: 13/12/2018

DH 18/13 (11-07-2013_16h35)
*DH 18/13 (28-02-2013_17h31)
(formerly DB23/05)

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DEGREE: MSc

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STUDENT NUMBER: 212253336

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(YES)	~~NO~~

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Chapter 1: General introduction

Hybridisation can be defined broadly as the transfer of genes between genetically distinguishable species (Rieseberg et al., 2000). The study of hybrid plants has a rich history. Scientific morphological identification of hybrids is thought to have begun with Cotton Maher who correctly identified *Zea mays* and *Cucurbita* spp. as being of hybrid origin in 1716. One of the early pioneers of the study of hybridisation was Joseph Gottlieb Kölreuter (1766), who's rigorous investigations into the consequences of hybridisation showed that early generation hybrids tend to be phenotypically intermediate between their parents while later generation hybrids tend to resemble their parents (Goulet et al., 2017). Kölreuter (1766) also demonstrated that hybrids from interspecific crosses may form sterile offspring. From this he concluded that hybrids are unlikely to form in nature without human intervention or disturbance. This was the first explicit mention to the importance of ecological factors in mediating hybridisation (Rieseberg and Carney, 1998). A modern pioneer of plant hybridisation studies is Loren Rieseberg. Over the last decade she has undertook nearly every topic related to plant hybridisation. This has included papers on the influence of hybridisation on plant morphology and molecular markers (Rieseberg et al., 2011), the impact of hybridisation on plant speciation (Rieseberg et al., 2000), the contribution of hybridisation to plants colonizing novel environments (Lexer et al., 2005), the origin of reproductive isolation in plants (Baack et al., 2005) and the consequences of hybridisation in plants (Rieseberg and Wendel, 1993).

A Web of Science search on plant hybridisation (January 1993– November 2018, keyword 'plant hybridisation') returned, 17720 papers on the topic. Most of the papers focused on Plant Science (>50%). The number of studies on plant hybridisation have increased over the last decade (Fig. 1.1). This is unsurprising as technology has improved dramatically over this time and provided the tools to explore hybridisation. This has allowed for increased numbers of studies in disciplines such as biochemistry, molecular biology, genetics/hereditary, biotechnology and evolutionary biology (Fig. 1.1). There was an spike in the number of studies on plant hybridisation in 2003. This could not be explained by a special issue on the subject in 2003. The technology to study plant hybridisation was readily available. This can be the only reason for this rise in studies in 2003. This number declined after 2003, but slowly began to increase again from 2008-2010 (Fig. 1.1).

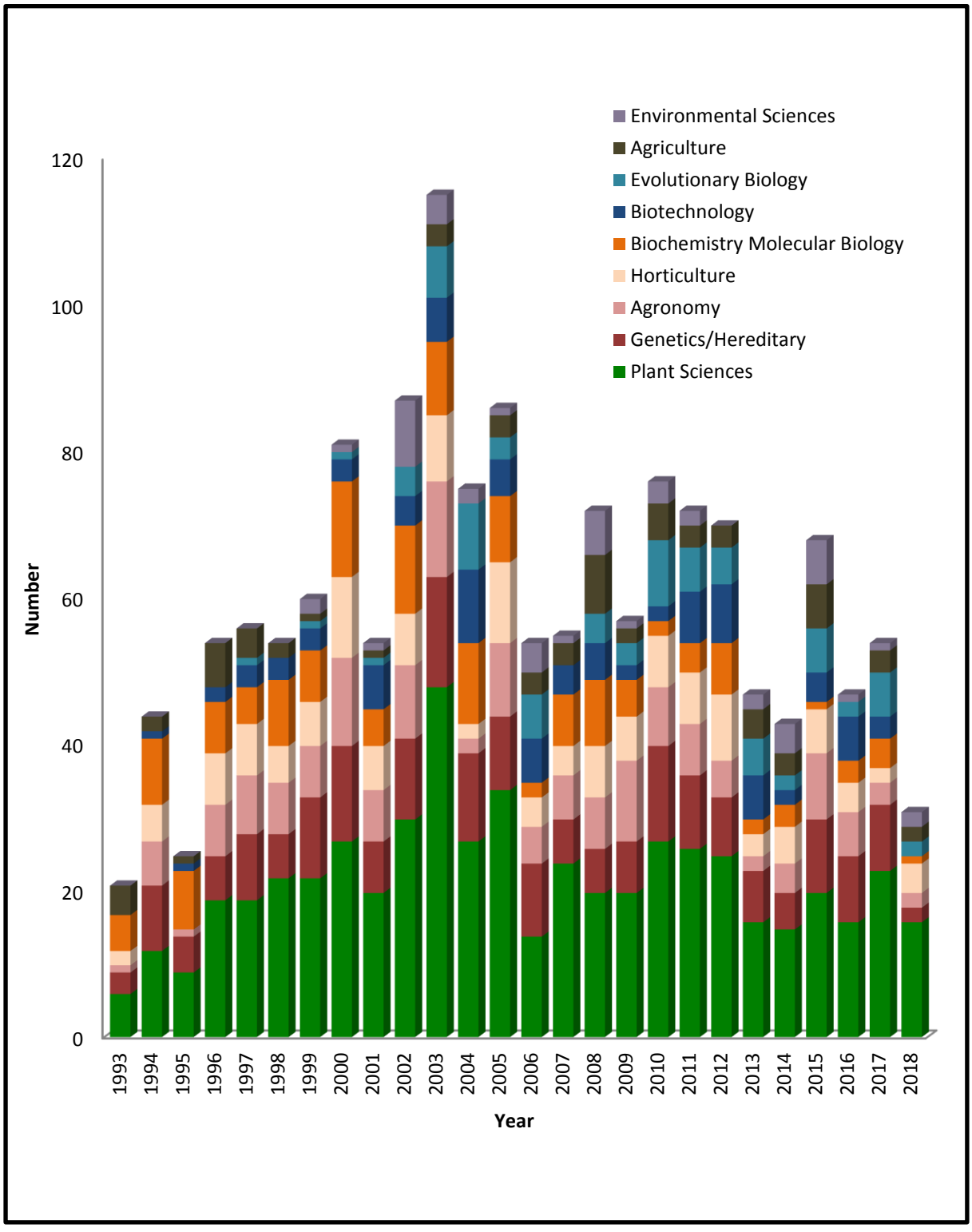


Fig. 1.1: A subset of publications retrieved from Web of Science database when using the search term: "plant hybridisation" divided into general categories

In South Africa, little progress has been made in documenting hybridisation brought about by humans relocating species outside of their locally endemic habitats despite its global

relevance and a large cut flower industry in the country (Rebelo, 2001). Hybridisation amongst wild members of *Protea* are has been rarely documented in scientific literature (Linder, 2003). Of the papers discussing Proteaceae and the genus *Protea*, the majority are focused on human induced hybridisation for cultivation— (Johnson and Briggs, 1974; Blomerus et al., 2010; Littlejohn, 2001) and only two studies focused on natural hybridisation between species (Liu et al., 2006; Mitchell and Holsinger, 2018).

Forms of hybrids in the Proteaceae were first termed 'Frankenflora' by Tony Rebelo (Rebelo, 2001, 2005). Frankenflora are hybrids that result in a loss of a species, and then become dominant community game changers. Rebelo (2001) wanted to emphasize, using the name, the monstrous potential impact that hybrid swarms could have on natural ecosystems if humans shift plants outside of their natural ranges. He considered these 'Frankenflora' to be a greatly misunderstood threat to the genetic diversity of local species (*sensu* Laikre et al., 2010). In this thesis, my focus is on the detection of 'unnatural' hybridisation of the *Protea* species in the eastern edge of the Cape Floristic Region. The study area, the Van Stadens Nature Reserve in the Eastern Cape, has documented introductions of *Protea susannae* E.Phillips species native to the Western Cape. There is known hybridisation between endemic *Protea eximia* (Knight) Fourc. and *P. susannae* at the reserve detectable by a unique sulphur smell, which is present in *P. susannae* leaves when they are crushed but nor present in *P. eximia* leaves. For the remainder of this introductory chapter, I discuss plant hybridisation and the genetic identification of plant hybrids. Plant isolating mechanisms specific to the Proteaceae are also discussed, as it is necessary to understand the methods which plant species use to prevent or reduce hybridisation. These barriers need to be absent for plant hybridisation to take place. Finally, examples of global 'Frankenflora-esque' occurrences are used to demonstrate that this is not a problem that is just restricted to the Cape Floristic Region.

Hybridisation in plants has been redefined many times in the past. Stebbins (1950) described hybridisation as being more than the sharing of genes between species (i.e. interspecific hybridisation). Interspecific hybridisation is part of a much more widespread phenomenon of gene sharing and replacement. Grant (1981) stated that the concept of hybridisation was the movement of genetic material between parental types, ultimately leading to the production of hybrids. This idea of hybridisation predates the genomic era. It was founded upon knowledge of phenotypic variation between plant species and hybrid offspring (Marsden-Jones, 1930). Harrison (1990) defined hybrids more broadly as the "offspring between individuals from populations which are distinguishable from one another on the basis of one or more heritable characters". Recently, Mallet (2005) described hybridisation in its simplest form: "a crossing of genetically distinguishable taxa, which leads

to the production of sexually viable offspring that contain and express both genetic and phenotypical information from both parents". Hybridisation, by reshaping the genetic composition of populations, has the potential to create novel genotypes that adapt to new environments (Stebbins 1950; Arnold and Hodges 1995). Thus, new species may arise, but so too, many existing ones may be lost.

Understanding the source of the hybridisation event occurring is necessary to defining the type of hybridisation taking place and determining its potential impacts. Hybrid plants are introduced into an environment via one of three routes: natural hybridisation between complementary parapatric native species, hybridisation between native and exotic species and the introduction of extralimital taxa into a natural environment (Vilà et al., 2000). The frequency of hybridisation events has dramatically increased due to human-mediated dispersal (Wolfenbarger and Phifer, 2000). As an example, *Corymbia* K.D.Hill & L.A.S.Johnson F_1 hybrids (*Corymbia torelliana* x *Corymbia citriodora*) are of great commercial importance to the Australian forestry industry (Dickinson et al., 2013). These hybrids are frequently translocated to new plantations far beyond their natural range due to their wide adaptability to the tropical conditions of parts of Australia and higher quality timber output compared to pure *Corymbia* species (Lee et al., 2009). The use of hybridisation in cultivation gives rise to commercially desirable qualities in the timber. But at what cost? In a subsequent study, Dickinson et al. (2013) determined that reproductive isolating mechanisms were found to be the weakest between *Corymbia* hybrids and wild stands of *C. citriodora*. According to Dickinson et al. (2012) the use of hybrid taxa in forest plantations can lead to gene flow from hybrid populations into native populations of *C. citriodora* near to the plantations. Thus, natural forests close to hybrid plantations are at a risk of genetic pollution (Lee et al., 2009), threatening wild *Corymbia* diversity.

The possibility of hybrid plants becoming invasive was first confirmed by Anderson and Hubricht (1938), who described that the process of introgression, or 'introgressive hybridisation'; as the transfer of genes between species due to repeated backcrossing. Their study focussed on hybridisation between *Tradescantia canaliculate* Raf. and *Tradescantia subaspera* Ker Gawl var. *typica* E.S.Anderson & Woodson. The two species co-occured in an area of erosion along a cliff face and then backcrossing took place. Hybrids of the two species were found wherever the habitats met. Backcrossing, occurs when a hybrid individual crosses with a parent or a closely genetically related species to the parent; an example of this has been documented between the German native *Rorippa sylvestris* (L.) Besser and the introduced invasive *Rorippa austrisaca* (Crantz) Besser (Bleeker, 2003). The hybrids were either intermediate or very close morphologically to *Rorippa sylvestris*. This is concerning as backcrossing during a full hybridisation invasion of *R. austricus* may not be

observed in field based on morphology. The identification of interspecific plant hybridisation where hybrids that are not morphologically distinct from their parents is only possible with the use of genetics. Thus the genetics era revolutionised such research as molecular markers could be used to identify hybrids amongst plant species.

Hybridisation can also be linked to invasiveness. Schierenbeck and Ellstrand (2009) used molecular data from a common invasive plant species to determine if invasiveness was linked to hybridisation. From this, they suggested that hybridisation is a potential stimulus for the evolution of invasiveness in plants, and that hybridisation preceded invasiveness (Schierenbeck and Ellstrand, 2009). These sentiments were reiterated by Gaskin and Schaal (2002), who found that the most common invasive plant in the USA was a hybrid of two *Tamarix* species— these parental species were originally geographically isolated in Eurasia.

The innovation and development of molecular genetics has come to be the cornerstone of the identification and determination of hybrid plant invasive potential, (Rieseberg and Ellstrand, 1993). Anderson and Hubricht (1938) identified hybrids by recording slight morphological differences in leaf, node and internodes between herbarium specimens of the American species, *Tradescantia occidentalis* (Britton) Smyth found within and outside the range of *Tradescantia canaliculate* Raf.(also an American species). Morphological comparison to identify hybrids in this case proved to be only successful when the percentage of *T. occidentalis* x *T. canaliculata* hybrids occured closer to the natural range of *T. canaliculata.* Morphological identification alone cannot provide other important information such as the direction of gene flow during hybridisation. This frustration of being unable to understand the inner mechanisms of plant hybridisation was highlighted by Anderson (1948). Biologists at the time only based interspecific hybrid identification on observable morphological clues in the field. Anderson argued that the biologists of his day would not be able to determine the true degree and extent of introgressive hybridisation until plant species could be genetically analysed to determine the spread of their marker genes. His ideas have come into fruition in the molecular era. For example, Repplinger et al. (2007) showed that three sympatrically occurring native species of Asteraceae hybridised naturally in central Germany. Some phenotypical differences were noted between the *Arctium* L. species and their putative hybrids, but this was not enough to tell them apart. The identification of natural hybridisation was only possible by use of the modern technology of randomly amplified polymorphic DNA (RAPD) markers. The guide to authors of *Molecular Ecology* 2018 had highlighted issues about the reliability of RAPD markers. As of 2018, we have access to overwhelming quantities of genomic data on taxa that was unimaginable to the early pioneers of the study of biological hybridisation. Just one of the advantages of the strides in technology we have made is to show that genomes, in some cases, are mosaics of

fragments of different ancestry (Song et al., 2002). This, it can be interpreted that the fragments of some plants are more restrictive to gene flow than others are. This would lead to the phenotypic consequences of hybridisation being displayed in some hybrid taxa and not others (Goulet et al. 2017; Mallet et al., 2016; Cruickshank and Hahn, 2014). The course of refining our understanding of hybridisation will continue to progress into the future as the unfolding development and improvement of new technological methods shapes how we identify hybrid plants from parent species.

As discussed above, some plant hybrids may be easy to distinguish from their parents due to distinct differences in leaf, flower or stem morphology but others may be cryptic, looking like one of the parental species. The identification of hybrids becomes further confounded when a hybrid crosses with another species (i.e. not one of the parent species). An example of this is the hybrid offspring produced by the cross between *Hibiscus dasycalyx* S.F.Blake & Shiller and hybrid *Hibiscus* 'Moy Grande'. *H* 'Moy Grande' contains a striking pedate (leaves resemble feet) leaf morphology very similar to the leaves of *H. dasycalyx* (Fig. 1.2). *Hibiscus* 'Moy Grande' is the hybrid of *Hibiscus grandiflorus* Michx. and *Hibiscus mocheutos* L. and contains an acuminate (shaped like an acute angle with an accentuated point) leaf shape (Fig. 1.2). The mean leaf length-width ratios were also far greater for *H. dasycalyx* than those for *H.* 'Moy Grande' (Yu et al., 2016). When looking at the floral anatomy, the flowers of the two interspecific hybrids species could not be easily determined from the floral structure colouration of the parent, *H.* 'Moy Grande' (Fig. 1.3). Genetic analyses capture the variation between the two parent taxa and their offspring. The analyses showed that there were genes of *H. dasycalyx*, *H. grandiflorus* and *H. mocheutos* within the offspring. In this case as molecular evidence was the only means to unravel the hybridisation history as the morphology was misleading (Yu et al., 2016).

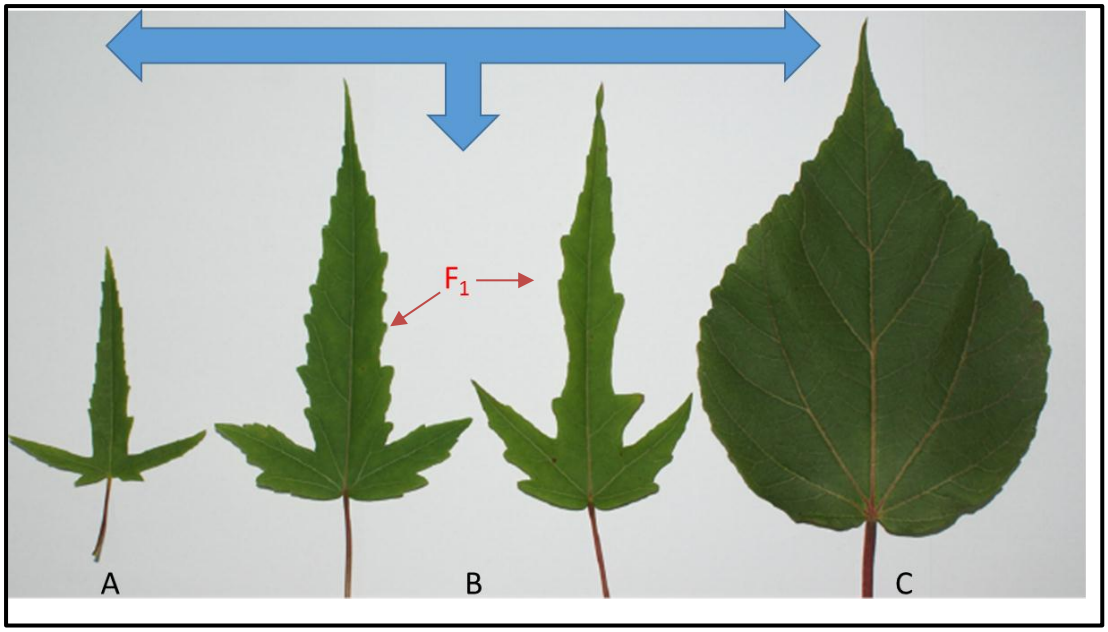


Fig. 1.2: Leaf morphology of **A**: *Hibiscus dasycalyx*, **B**: Two F_1 interspecific hybrids produced from the cross and **C**: *H.* 'Moy Grande' (Adapted from Yu et al., 2016).

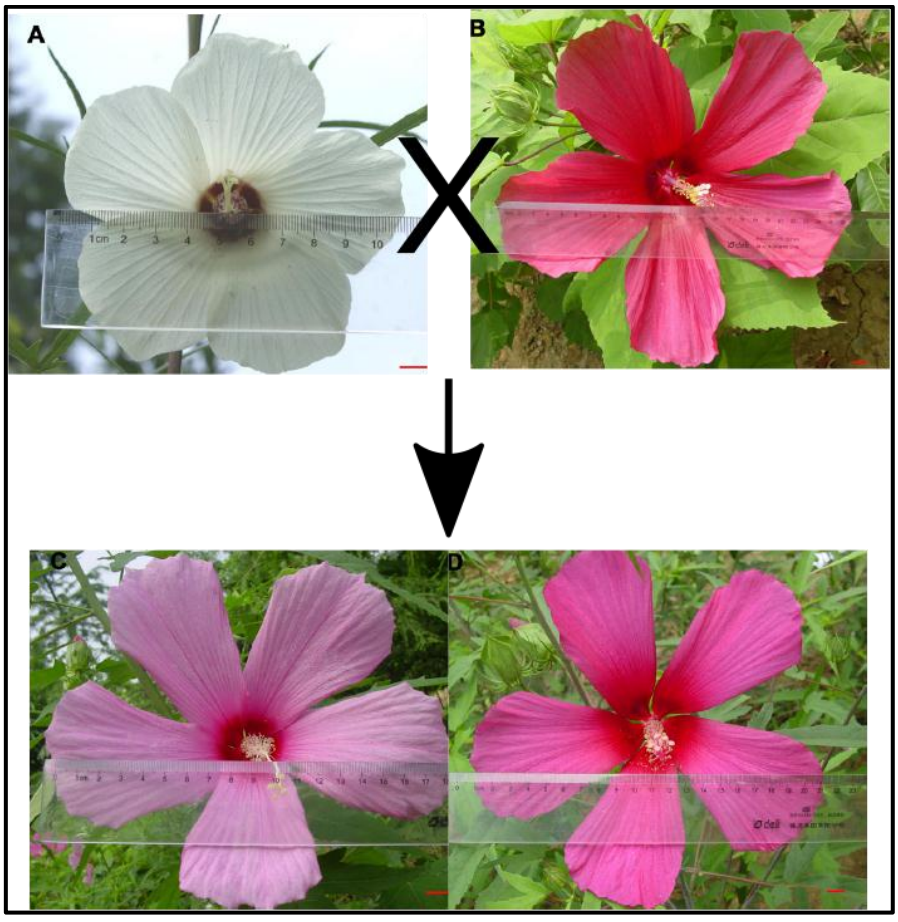


Fig. 1.3: Floral morphology of **A**: *Hibiscus dasycalyx*, **B**: *H.* 'Moy Grande' and **C & D**: Two interspecific hybrids produced from the cross (Adapted from Yu et al., 2016).

Hybrids can also have novel morphological characteristics rather than a mosaic of direct parental morphological characteristics (Gottlieb and Ford, 1988). Reiseberg and Wendell (1993) compared the predictability of parental plant genetic characteristics to be passed on to hybrid offspring to the likelihood of morphological traits to be passed on in the same cross. They assumed that morphological differences between species arise because of structural genetic differences between species. The crossing of two species would then produce hybrids with a blend of the parental characteristics as well as the rise of new novel characteristics. The high percentage of parental characters found in F_1 hybrids would allude to the idea that presence or absence, structure and quantality of these characteristics are all administered by a single dominantly inherited gene (Hilu, 1993). Over time, with the development of F_2 and F_3 hybrids in plants, simple genetic control leads to the development of a mosaic of intermediate characteristics. These insights were first discovered, famously, by Gregor Mendel in his pea experiments of 1866 (Thomas et al.,1996). Similarily, the results of Reiseberg and Wendell (1993), found that 64% of F_1 and 89% of later generation hybrids display novel morphological characteristics that were not inherited from the initial cross of parent species. Thus, hybrids can be very difficult to identify from morphology alone and hybrids can be misidentified as new species (e.g. Walker et al., 2018)

Hybridisation events are often facilitated by long distance dispersals of a species from one habitat to another. Long distance human-mediated dispersal of *Spartina alterniflora* Loisel, an American endemic, to France has led to hybridisation events where previously allopatric species of *Spartina maritima* (Curtis) Fernald encountered it (Baumel et al., 2003). Only some hybrids could be identified through morphology, while the true extent of the introgression was unknown (Marchant, 1977). The ecological and evolutionary consequences for these interactions need to be further understood to prevent the costs of a biological invasion. Introgressive hybridisation on this level may lead to the loss of *Spartina maritima*. Baumel et al. (2003) showed that traces of *Spartina alterniflora* could be found in plants growing in French estuaries using ISSR molecular fingerprinting however, recently *Molecular Ecology* Guide to Authors, 2018 highlighted reliability issues of ISSR data. In both the studies, the molecular techniques of hybrid identification could show a much clearer ecological pattern to the introgression of *S. alterniflora* into *S. maritima* than morphological identification alone. Another example of this was the introduction of *S. alterniflora* along the British coastline (North Wales), *S. alterniflora* hybridises with *S. maritima* forming the sterile hybrid *S.* x *townsendii* (Thompson, 1991).

Why should we care if plant species hybridise? Is hybridisation not a natural evolutionary process? Or are knock-on effects of interspecific hybridisation an unavoidable consequence

of a human-fractured environment? Many plant geneticists have asked the same questions and there are polarised views with regards to the future role of hybridisation to the conservation of plants. Table 1.1 is a summary of the views of some of the influential voices of the topic of plant hybridisation.

Table 1.1. The positive and negative impacts of plant hybrids

1.) Positive	2.) Negative	References
Establishment of species with greater genetic diversity	Breeding offspring prone to invasiveness	1.) Gramlich et al. (2016) 2.)Ellstrand and Schierenbeck (2000)
A transfer of adaptations between species	A loss of adaptation as genome is invaded	1.) Rieseberg and Wendell (1993) 2.) Mallet (2005)
Rapid and repeatable mode of speciation	Hybridisation reduces fitness of offspring	1.) Reiseberg et al. (2000) 2.) Keller et al. (2000)
Hybridisation leads to the origin of new taxa	Increase in the extinction rates of rare species	1.) Abbott (1992) 2.) Wolf et al. (2001)

There are four possible outcomes for a species facing the threat of hybridisation (Fig. 1.4). The incompatibility mechanisms in place may prevent hybridisation from occurring or the hybrid that forms may produce sterile offspring. This prevents the formation of hybrids or outbreeding depression, the decreased fitness of the hybrid offspring may occur (Petit and Excoffier, 2009). Lastly hybridisation may lead to heterosis, this is the increase in biotic superiority in hybrid offspring compared to their parents (Oakley et al., 2015). The hybrids that form from the hybridisation event will have increased vigor and improved biological functions (Kearney, 2005). With these enhancements it can outcompete its non-hybrid counterparts.

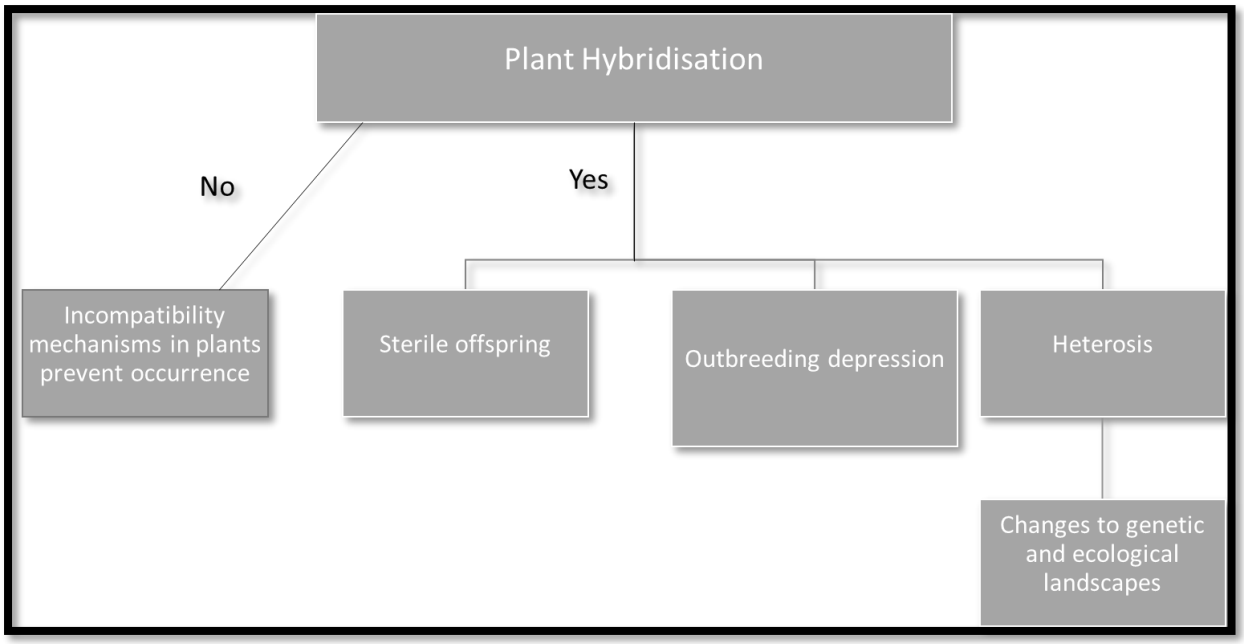


Fig. 1.4: The potential outcomes plant individuals face following any form of hybridisation event. Figure adapted from Rieseberg et al. (2000) and Levin (1978)

Opposing views of how hybridisation may effect local plant populations (Table 1.1) can lead to complicated environmental conservation decision making (Whitham et al., 1999). This is especially the case when considering the conservation of rare and near-threatened populations (Frankham et al., 2011). The title of López-Pujol et al. (2012) asks a defining question in this area of plant conservation: “Should we conserve pure, hybrid species or both?” And the answer is not simple, as discussed by López-Pujol et al. (2012). Genetic swamping of rare species can lead to a pure species being assimilated by closely related hybrids. Genetic swamping happens when a local gene pool of becomes introgressed with domesticated or introduced non-native genes of the same species and the entire genetic diversity of that local species is lost (Hufford and Mazer, 2003). Genetic swamping is very rarely caused by natural hybridisation (Riseberg and Ellstrand, 1993). It is far more often caused by a human introduction of a foreign species into a habitat (Petit et al., 2004). On the other hand, hybridisation may increase the genetic diversity and fitness of rare individuals. This is a possible side-effect of heterosis (Jinks, 1983). However, there is a very fine line between the genetic survival adaptations inherited by a species during a hybridisation event and the loss of genetic diversity that occurs due to gene introgression. Very few papers currently discuss this important issue.

Other studies focus more on the negative impacts of hybridisation on local endemics. For example Keller et al. (2000) examined the impact of introducing non-local seed mixes to local farmland to restore biodiversity. The study showed that the seeds from the United States (US), British and German *Agrostemma* L. and *Papaver* L. species began hybridising with local Swiss wildflowers of the same genera, and F_2 backcrosses formed overtime. The survival rate of these hybrids was much lower than that of the parent species. Thus, these authors suggested that only the seeds of plants of local origin should be used when attempts are made to improve local biodiversity. Holderegger (1998) agreed with these findings. He showed that species such as *Saxifraga aizoides* L. and *Saxifraga mutata* L., that occurred in differing habitats on an alpine slope, could readily hybridise due to weak hybridisation barriers between them and narrow, but sympatric, habitat requirements of both species. An increase in slope erosion was found to be a major driver on the landscape dynamics of these species that led to the decline in hybridisation barriers between the two species.

With the potential negative impacts of introducing foreign plant genotypes, including genetic swamping, care must be taken with crop and plant translocation (Hufford and Mazer, 2003). We, as conservation biologists, need to determine areas that plants can be moved to for further financial gain, but with little or no consequence on genetic fitness of local populations. Introduced populations may hybridise with local ones, which may lead to unintended and unfortunate consequences (Fenster and Galloway, 2000). A further example of this is exemplified in the study of Larcombe et al. (2013), who showed that expanding *Eucalyptus* plantations may threaten native *Eucalyptus* L'Hér. species if the habitat of the proposed plantations is not properly assessed. This was true with *Eucalyptus ovata* Labill. which had not previously encountered *Eucalyptus globulus* Labill. in Victoria and Tasmania. Geographic surveys were conducted to ascertain wildling establishment of *E. globulus* introduced from industrial *E. globulus* plantations. The expansion of *E. globulus* plantations into these provinces can be directly linked to the 1% increase in morphological identification of hybrid seedlings. This study also showed that after six years 68% of the *E. globulus* x *E. ovata* hybrid seedlings were less likely to survive than pure *E. ovata* seedlings. In the last two decades in Australia there has been a dramatic increase in the area and distribution of *Eucalyptus* plantations. The increased expansion by plantation owners and the lack of reproductive isolation leads to fears by Australian ecologists of genetic pollution to natural *Eucalyptus* populations (Potts and Dungey, 2004). Another example is morphological identification of hybrids of the rare Tasmanian species *Eucalyptus perriniana* F. Muell. ex Rodway. encroached upon by plantations of exotic *Eucalyptus nitens* H.Deane & Maiden, for which a risk assessment revealed hybridisation was possible because of pollen flow

between *E. nitens* and *E. perriniana* was a well-documented event, which is predicted to be exacerbated by climate change (Larcombe et al., 2012).

This further confirms the view that anthropogenic movement of plant species into new habitats, for economic gain has a strong potential of resulting in unintended hybridisation between local and extralimital species. Olden et al. (2004) suggests that human-assisted dispersal of plants substantially increased the probability that pairs of interacting species could hybridise. At the same time, human-mediated habitat disturbance provides environments suitable for hybrid progeny to thrive in Kramer and Havens (2009).

If the consequences of hybridisation are so dire for plants and the surrounding environment (Levin et al., 1996; Rhymer and Simberloff, 1996; Vilà et al., 2000; Allendorf et al., 2001), then how is hybridisation usually prevented? Why do not all plants hybridise? The simple answer is that plants have a genetically determined set of isolating mechanisms in place that are supposed to prevent the spread of genes from one closely related species to another. This set of mechanisms are divided into two broad categories: Geographic and Reproductive isolating mechanisms (Fig. 1.5).

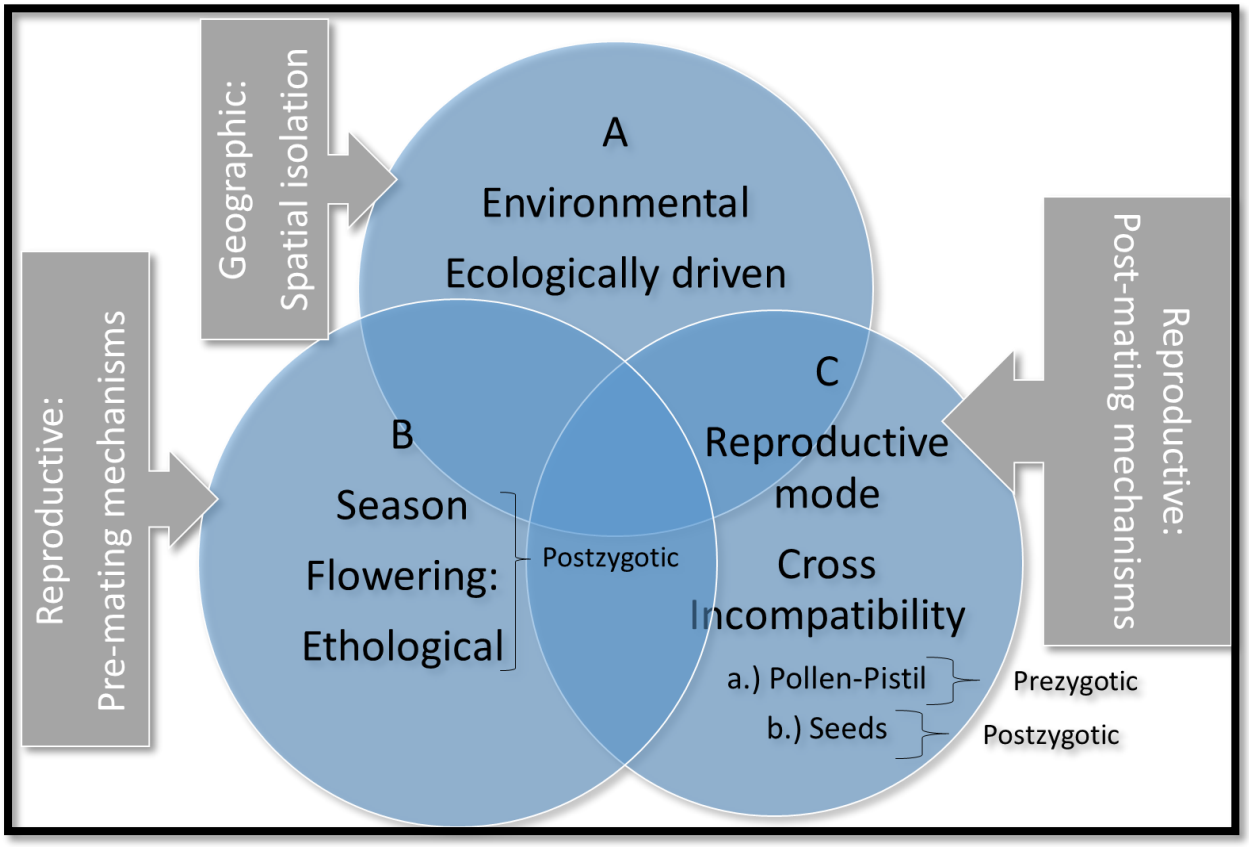


Fig 1.5: Representation of the different co-occurring isolating mechanisms utilised by plants to reduce the possibility of hybridisation (adapted from Levin, 1978).

The role of geographic isolating mechanisms as a foremost factor in the speciation of plant species is a contested one. This ranges from the view that geographic barriers have a greater role to play than reproductive barriers (Davies et al. 2004; Vitousek et al., 2010; Verboom et al. 2015) to the opposite (Midgley and Bond, 1991; Lowry et al. 2008). In Baack et al. (2015), support for geographic isolation theory was backed up. The geography of the speciation process was seen to affect the degree of gene flow between diverging populations. The pattern of local adaptation and the nature of the selective forces at work also played a role. Increased speciation and reduced instances of hybridisation occurred to greater degrees if the potential parents were found to be allopatric and found in different habitats. The change in habitat is too costly for an immigrant species to flourish and begin to hybridise with similar species (Rundle and Whitlock 2001). This conjecture is obviously species specific and will differ with the diverse reproductive methods of the dissimilar species. Seehausen et al. (2008) supports the idea that a lack of heterogeneous habitats leads to greater species homogenisation and increased occurrences of hybridisation between similar species. Their study recorded the weakening of heterogeneity between biomes over time. This facilitated and encouraged introgressive interspecific hybridisation between divergently adapted plant and animal species. Lexer et al. (2013), argued for the geographical isolation of plant species as a major driver of species maintenance and diversity in the Cape Floristic Region. The mountainous topography and varied regional climates and soils restricts the ranges of the natural Fynbos vegetation. This argument was also referred to in Cowling et al. (2003), where the position of upland-lowland gradients in the Fynbos biome were shown to be where the largest pool of genetic material for species to diversify was located. They exist and have remained as cohesive units in their specific habitats for millennia (Macpherson, 2017). More evidence of geographic isolation playing a role in Fynbos species can be found in the example of *Protea repens* (L.) L which was found to be isolated by habitat and by distance from closely related family members (Prunier et al., 2017). Then the obvious question is: what would happen if someone were to move one of these species from one of these unique subunit habitats to another? Would there be sufficient isolating mechanisms in place to prevent closely related taxa from interbreeding? Are geographic reproductive isolating barriers too weak to prevent the hybridisation of sister species? These are just three of the many questions put forward that this thesis seeks to answer in a limited context using the Van Stadens Wildflower Reserve as a case study area.

If geographic barriers cannot prevent or slow down hybridisation of two closely related taxa then the alternative is that reproductive isolation barriers or mechanisms may do so (Fig. 1.5). As with geographic isolation, there are many studies that have focused on the role of reproductive isolating mechanisms to maintain species richness and diversity. Reproductive isolation is divided into two major categories: pre-mating and post-mating mechanisms. Pre-mating mechanisms prevent pollination from occurring. The post-mating mechanisms are themselves subdivided into two groups: pre-zygotic and post-zygotic mechanisms. The pre-zygotic mechanisms focus mainly on ways to prevent pollen from a different species from accessing the stigma. These mechanisms generally act at the stage of flowering for all involved (Kay, 2006). Post-zygotic mechanisms only occur if the pollen remains viable past pre-zygotic controls. Post-zygotic mechanisms act after pollen and ovule fertilization between two non-compatible species has already taken place. It ensures the seeds produced are sterile and leads to total reproductive isolation of the potential offspring produced (Nosil et al., 2005). Lowry et al. (2008) assessed the breeding of 40 separate crosses between 19 closely related pairs of taxa. They found that pre-zygotic isolation was twice as likely to prevent F_1 hybrid formation than post-zygotic isolation. The pre-zygotic barriers were weak enough in four of the taxa to form hybrid offspring. Larcombe et al. (2016) showed that in many of the crosses between *Eucalyptus nitens and E. globulus*, the pre-zygotic or mechanical barriers was severely reduced or lost entirely. The prezygotic barriers include habitat isolation, gametic isolation or mechanical isolation (Servedio and Sætre, 2003). Post-zygotic barriers reduce the capability and capacity of hybrids to produce offspring. This can include sterility of the offspring (Rieseberg and Willis, 2007). In contrast to the pre-zygotic barriers, the post-zygotic barriers persisted in the hybrids (Larcombe et al., 2016). This may be species-specific to *Eucalyptus*. However, a concerning finding by Lowry et al. (2008) was that of the attempted crossing of the 40 plant pairs; 22.5% of the crosses produced viable F_1 hybrid seedlings. This shows that in certain cases both pre-zygotic and even the post-zygotic barriers can be bypassed.

Then there is the contention that we should approach the theory of geographic isolation of species with thoughtful care (Baack et al., 2015). The geography of the speciation process affects the degree of gene flow that occurs between diverging populations. Pollen dispersal and seed flow play a great role in maintenance of unique species (Kendrick, 2012). For example different *Clarkia* Pursh populations separated 1-3 km from each other were defined as reproductively isolated (Runquist et al., 2014). On the other-hand *Petunia* Juss species populations were only considered isolated when they were found at 20 km from each other

(Dell' Olivo et al., 2011). This leads to us questioning what can be considered a range overlap? Does the type of species play a role in this? If species can disperse their genetic material across long ranges and bypass geographic barriers, then these barriers are not as effective a measure against hybridisation as previously thought.

Baack et al. (2015) and Levin (1978) differ from most of the other papers I have reviewed thus far explaining the conservation of diversity and prevention of hybridisation by flora in the Cape Floristic Region and across the globe. Other papers either took one side or the other (ie: Geographic vs Reproductive modes of isolation) as the main drivers of diversity. These two papers, with a 37 year gap between them of , highlighted the importance of viewing geographic and reproductive isolating barriers as overlapping concepts. Levin (1978) noted how reinforcement of reproductive barriers can take place between species that have weak temporal and environmental barriers. Moreover, he states that species that have arisen through catastrophic selection and chromosomal reorganization will be more cross-incompatible to related species than population-rich species that diverged gradually via geographic isolation. A connection can be drawn from this statement and the *Proteaceae* family that diverged gradually due to geographic isolation in the Cape (Prunier and Holsinger, 2010). Baack et al. (2015) stated that: "reproductive isolation, whether partial or complete, is the essential cause of plant diversity." The effect of distance on geneflow depends on the method of dispersal. Wind and bird dispersed species require much greater levels of distance to achieve the same isolation than bee dispersed, or reproductively isolated species achieve at 100 m from each other (Baack et al., 2015).

The Proteaceae have many documented pathways of maintaining reproductive isolation. Several *Banksia* spp are noted for their asynchronous flowering times as a method of reproductive isolation. In the same vein some *Protea* spp. produce a low seed set if they hybridise (Table 1.2). In the Cape Floristic Region (CFR) reproductive isolating barriers are weakened by strong geographic barriers that are in place. This idea is alluded to in Latimer's Neutral theory of the fynbos biome (Latimer, 2014). The fynbos biome is compared with the Amazon and is shown to have a higher rate of diversity with plant migration rates that are two orders of magnitude lower than in the Amazon. The Amazon is dominated by rare plant species that have large ranges. The CFR, on the other hand, is dominated by narrow ranged endemics. The neutral theory supports the idea that the CFR communities are topographically fragmented. They are islands separated not by water, but by drier lowlands or higher mountains. This is the geographic isolation that drives diversity in the CFR (Goldblatt and Manning, 2002). This geographic isolation may have led to weak reproductive barriers and hybridisation may be a consequence of the reconnection of species that have been isolated for long periods of time (Cowell, 2005).

Table 1.2: Isolating mechanisms compared in the Proteaceae (adapted from Baack et al. (2015)).

	Barrier Type							
	Geographic		Reproductive					
Species	Environmental	Ecological	Flowering time	Pollen Pistil-Cross Incompatibility	Different methods of Reproduction	Seed Sterility	Hypothesis	Citation
Banksia prionotes Lindl.	X	X	X				Asynchronous flowering times are the main mechanism.	Lamont et al. (2003)
Banksia hookeriana Meisn.	X	X	X				Asynchronous flowering times are the main mechanism.	Lamont et al. (2003)
Leucadendron coniferum (L.) Meisn.		X		X	X		Wind pollination followed by pollen incompatibility	Rebelo (2001); Friedman and Barrett (2009)
Protea laurifolia Thunb.			X				Asynchronous flowering times are the main mechanism.	Rebelo (2001)
Protea aristata E.Phillips		X				X	Hybrids produce a low natural seed set and poor seed germination occurs.	Brits (1990)
Grevillea banksia R.Br.				X			Pollen tubes burst when come in to contact with incompatable pollen	Herscovitch and Martin (1990)
Conospermum taxifolium C.F.Gaertn						X	Offspring of *C. taxifloium* x *C. ellipticum* do not produce fruit and seeds are sterile	Morrison et al. (1994)

Lamont et al. (2003) documented that *Banksia prionotes* and *Banksia hookeriana* can hybridise if there is sufficient human disturbance to break the naturally occurring isolating mechanisms. Hybrids are often associated with disturbed habitats (Anderson, 1948, Ellstrand et al., 1999). Disturbances foster co-colonization of normally allopatric species, which creates habitats that hybrids can thrive in. Hybrids with superior competitive abilities may gradually replace parent species depending on the abundance of parent species and the available opportunities for hybridisation (Levin et al., 1996; Bleeker and Hurka, 2001).

Hybridisation in the Cape Florisic Region (CFR)

Hybridisation is known to occur in a few endemic plant families and genera in the CFR. These include the Apocynaceae (*Microloma* R.Br., Bruyns and Linder, 1991); Ericaceae (*Erica* L., Oliver, 1991); Geraniaceae (*Pelargonium* (L.) W. Aiton, Bakker et al., 1998); Proteaceae (*Leucadendron* R.Br. , Williams, 1972; Brits and van den Berg, 1991 and *Protea*, Littlejohn et al., 2001) and Rosaceae (*Cliffortia* L., it is proposed as the fundamental cause of the diversity found within the genus, Whitehouse, 2002).

The CFR of South Africa contains a very high diversity of endemic plant species. This may be as a knock-on effect of the historically low extinction rates of the CFR, or by contrast, due to stable environments over time which lead to high rates of ecological speciation to occur (Van der Niet and Johnson, 2009; Cowling et al., 2015). The CFR landscape contains infertile soils with fire-adapted, low-lying sclerophyllous (scrub) plants (Cowling et al., 1996). A possible factor that has maintained the high diversity of species in the region has been the adaptation of low seed dispersal distances in long persisting isolated populations (Prunier et al., 2017; Schluter and Pennell, 2017). However, this very key environmental adaptation can become misappropriated when species translocations occur. Geographic isolation may mean weak reproductive isolating mechanisms between related species.

Verboom et al. (2016) showed evidence of natural hybridisation between 68 of the current 84 species of *Jamesbrittenia* Kuntze using phylogenetic relationships between the species.

They demonstrated that the different species could cross readily and produce viable offspring. Geography was shown to be an important factor in maintaining species diversity. It was further determined from the morphology of species with overlapping ranges that hybridisation had occurred. This important role of geography as a factor in maintenance of species identity is a feature that needs to be kept in mind when species are translocated.

Introgression can be a problem when it blurs the natural boundaries between species. A case of introgressive hybridisation in a hybrid zone in the Swartberg Pass was documented between *Psoralea sordida* C.H. Stirt. & Muasya and *Psoralea forbesiae* C.H.Stirt., A.Bello & Muasya (Bello et al., 2018). The results showed that *P. sordida* and *P. forbesiae* are distinct species that are interconnected by a range of intermediate hybrids. There were so many hybrid offspring that the population sampled could be referred to as a hybrid swarm. Disturbance by road building was suggested to drive the development of the hybrid swarm in the habitat. The study of hybridisation is both current and urgent if we are to understand the breakdown of pre-zygotic and post-zygotic barriers in plants and the threats of human-mediated long distance dispersal of species across the Cape.

Visser (2005) produced a morphological and genetic study on hybridisation between two *Protea* species; but as an unpublished Honours treatise. This study documented the reputed hybridisation between the locally endemic *Protea lepidocarpodendron* L. and recently introduced *Protea neriifolia* R.Br. at Silvermine Nature Reserve on Table Mountain. There is only a slight overlap between the natural ranges of these two species. Their natural range boundaries have never crossed within the Silver Mine Nature Reserve (Rebelo, 2001). Hybrids and 'pure' parental species were distinguished using both morphological and genetic characteristics. The morphological data showed that capitulum length was the best discriminator between *P. lepidocarpodendron* and *P. neriifolia*. The capitulum length of *P. neriifolia* was much longer than that of *P. lepidocarpodendron*. Changes in colouration of lower involucral bracts, involucral bract tips, upper involucral bracts and upper bracts of past seasons' flowers measured in a chi square test provided further discrimination between species and hybrids. The genetic data used ISSR variation and detected a high level of hybridisation in the Silvermine Nature Reserve. This was the first study to document that *Protea* species boundaries can be permeable and took issue with the planting of wild *Protea* congeners close to indigenous species, going so far as to see it as a threat to conservation of the *Proteaceae*.

There are more than 360 species in the Proteaceae, but more than one third of them are listed in Red Data Book for plants (Rebelo, 2001). Flowers from the Proteaceae family form a key part (60%) of the cut flower industry in South Africa (Reinten et al., 2011). *Protea*

species grown in southern Africa are exported mainly to Europe. Demand for *Protea* flowers with the perfect aesthetical features is especially high during the European winter (September to February). Cultivators attempt to increase productivity of hybrid *Protea* species to meet this high international demand (Gerber et al., 2001).

The Van Stadens Wildflower Reserve (Fig. 2.1; Fig. 2.2a) offers a model system for studying potential hybridisation and introgression rates within a range of Proteaceae species due to a history of multiple introductions starting in 1984. The locally occurring species of *Protea* on the reserve are *Protea cynaroides* (L.)L., *Protea eximia* (Knight) Fourc., *Protea foliosa* Rourke, *Protea lorifolia* (Salisb. ex Knight) Fourc., *Protea mundii* Klotzsch, *Protea neriifolia* R.Br., and *Protea repens* (L.) L, while the following species have been introduced beyond their natural range: *Protea compacta* R.Br., *Protea coronate* Lam., *Protea laurifolia* Thunb. and *Protea susannae* E.Phillips.

Conclusions

There is an apparent lack of published studies on Cape on hybrids formed due to species translocation and having their ranges shifted into the ranges of other species. This may be due to the perceived idea that plant geographic and reproductive isolating barriers are well developed and can prevent hybridisation. This thesis is a response to the lack of literature on 'Frankenflora', and it serves as an example to show the impact of human-induced hybridisation over time on a natural system.

Chapter 2: Re-opening the case of Frankenflora: Evidence of hybridisation between local and introduced *Protea* species at Van Stadens Wildflower Reserve

2.1) Introduction

To date there is one example of a genetically-confirmed hybridisation event in a Cape Fynbos plant lineage, namely that of — *Centella. triloba* × *C. macrocarpa (*Schubert et al., 1996). This, and the general lack of morphological documentation, gives the impression that reproductive barriers are well-established in Cape species (Potts, 2017). Gail Reeves' PhD on Protea phylogeny was strongly suggestive of *Protea* hybrids. The Protea Atlas Project identified (based exclusively on field observations) many putative hybrids. This includes examples of naturally-occurring hybrids (Mitchell and Holsinger, 2018), but also many between local and non-local species across a range of settings (e.g. nature reserves, escapees from botanical gardens, and roadside rehabilitation). Despite this long list of suspected hybrids within *Protea*, highlighted more than a decade ago, there has been no further research to formally identify hybridisation events (i.e. using morphological or genetic data). Phenotypic observation and phylogenetic studies have made brief mention of potential hybridisation events, but these lack the population level sampling required to detect recent (i.e. within the last few generations) hybridisation (Valente et al., 2010; Prunier and Latimer, 2010). Mitchell and Holsinger (2018) used restriction site associated genotyping to identify natural hybridisation between *Protea punctata* Meisn and *P. venusta* Compton in the Swartberg Mountain range. More than a decade ago, Tony Rebelo (Rebelo, 2001; Rebelo, 2005) warned that *Protea* hybrid swarms were developing due to species being translocated outside their natural ranges — he termed such hybrids "Frankenflora" and considered these a threat to the genetic integrity of local species (*sensu* Laikre et al. 2010). Here I report an example of this demonstrating gene exchange between *Protea eximia* (Salisb. ex Knight) Fourc. (a local species) and *Protea susannae* E. Phillips (a non-local species) in the Van Stadens Wildflower Reserve.

The Van Stadens Wildflower Reserve (Fig. 2.1; Fig. 2.2a) offers a model system for studying potential hybridisation and introgression rates within a range of Proteaceae species due to a history of multiple introductions starting in 1984. This study is focused on *P. eximia* (which is a widespread species that also occurs on the nearby Lady's Slipper Mountain; Fig. 2.1) and *P. susannae (*native to the De-Hoop and Cape Agulhas regions of the Western Cape). In

1984, *P. susannae* was planted in an orchard along one edge of the reserve (Fig. 2.2b); the translocation of this species to the Van Stadens Wildflower Reserve represents a long-distance dispersal event of over 600 km. *Protea eximia* and *P. susannae* are relatively easy to identify in the field due to substantial differences in morphology and odour.

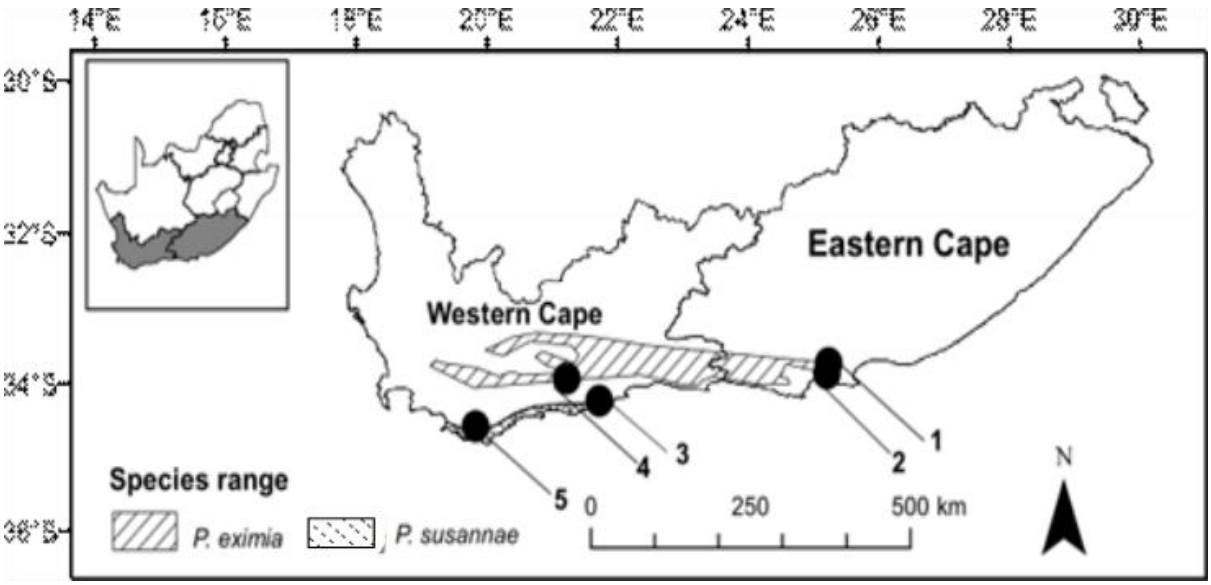


Fig 2.1. Map of the natural distributions of *Protea eximia* and *Protea susannae* and locations of study areas (1: Lady's Slipper mountain, 2: Van Stadens Wildflower Reserve, 3: De Hoop, Overberg District, 4: Garcia Nature Reserve, 5: Cape Agulhas)

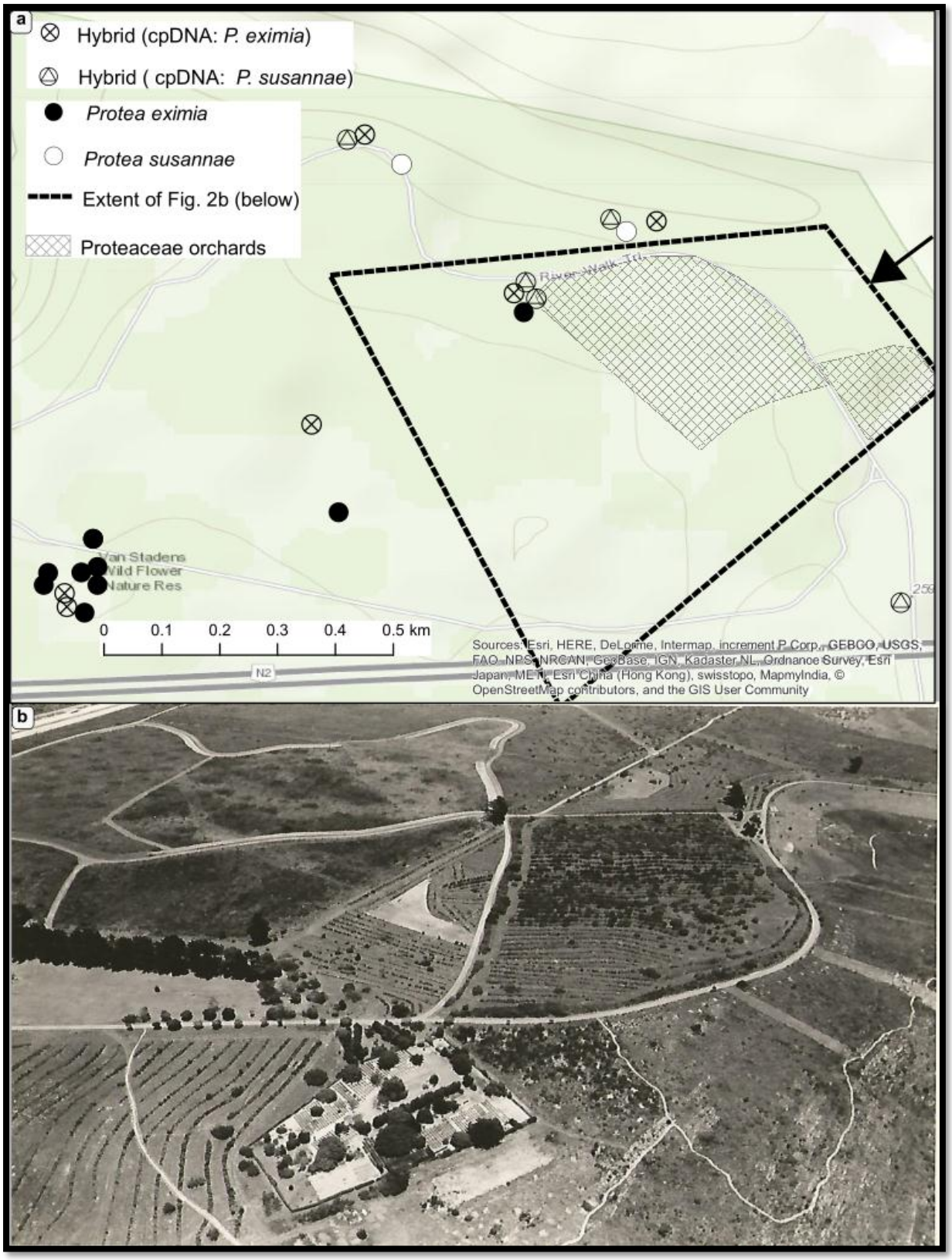


Fig 2.2. (a) Distribution of plants of *Protea eximia* and *Protea susannae* sampled in the Van Stadens Wildflower Reserve (the arrow indicates direction at which Fig. 2.2b was taken). (b) Aerial photograph taken in 1984 when *P. susannae* was first introduced to the reserve. Note

that no individuals were sampled in the original orchards (*P. susannae*) as this area had recently burnt prior to the February sampling.

Here I used morphological (leaf characteristics), odour (distinctive sulphur smell present in *P. susannae* leaves), nuclear and chloroplast DNA data to explore hybridisation between these two species; this confirms field observations of hybridisation documented in the Protea Atlas Project. The nuclear DNA data were obtained from the high-copy ITS region of the 35S rDNA cistron (the 5.8S rDNA and the flanking internal transcribed spacers, ITS-1 and ITS-2; hereafter referred to as ITS). The 35S rDNA cistrons form a multigene family arranged in tandem arrays that are confined to one or several chromosomal loci, the nucleolus organizer region(s) (NOR; reviewed in Volkov et al. 1999), each comprise hundreds to thousands of copies (Rogers and Bendich, 1987; Hemleben, et al. 1988). As such, evidence of recent introgression (i.e. hybridisation) will be stored in multiple areas of the genome and thus it is highly unlikely that a non-hybrid signal could arise via (Mendelian-like) allele sorting — even ancient hybridisation events can be etched into the ITS array (e.g. Grimm and Denk, 2007; Mahelka and Kopecký, 2010). Thus, hybrids will give rise to intra-individual site polymorphisms (termed 2ISPs, pronounced “twisps”; see Potts et al., 2014) in ITS; these 2ISPs should be readily evident in sequence chromatograms (Appendix B), even after multiple backcrosses of a hybrid lineage back into a population of the parent species. The chloroplast DNA was obtained from the 3’trnV $^{(UAC)}$ region (Shaw et al., 2014). As chloroplast DNA is usually inherited maternally from parent to offspring in most plants (Rhymer and Simberloff, 1996). In some Gymnosperms chloroplasts are paternally inherited (Hansen et al., 2007). it can be used as an informative tool to track the origin and direction of gene flow during hybridisation events. We use these genetic data to: a) confirm the occurrence of hybridisation between *P. eximia* and *P. susannae*, b) determine the direction of hybridisation (i.e. unidirectional or bidirectional gene flow), and c) compare with morphological indicators (including odour) of hybridisation to assess their consistency to detect hybrids.

2.2) Materials and Methods

Leaf samples from 24 plants of *P. eximia* or *P. susannae* — including plants identified in-field as potential hybrids due to mixed leaf and flower morphologies — were collected from the Van Stadens Wildflower Reserve in February 2017. These samples were meant to serve as a preliminary screening dataset to characterise the study system in terms of genetic and morphological variability. However, due to a wildfire on the 10th of June 2017, the vegetation on the entire reserve and surrounding areas was burnt and no individuals with leaf material remained. Thus, this study presents evidence of hybridisation but more extensive

geographic sampling on the next generation will provide a means to estimate the rates of introgression between these species.

To assess morphological and genetic variation within the context of the broader species' distributions, samples of assumed 'pure' (i.e. non-hybrid) individuals were collected: *P. susannae* from Cape Agulhas (n=8) and several localities east of the De Hoop Nature Reserve (n=10) and *P. eximia* from Lady's Slipper (n=6) and the Garcia Nature Reserve (n=3; Fig. 2.1). DNA extraction used an adaptation of the Doyle and Doyle (1987) protocol (described in Appendix A). Nuclear DNA sequence data were obtained from a single locus: ITS (described in the Introduction). To identify intra-individual site polymorphisms (2ISPs) indicative of hybridisation — as these may also occur due to intra-species variation (see Potts et al., 2014) — the ITS alignment and assumed 'pure' samples from the respective species were used to identify potential barcoding sites (i.e. substitutions exclusive to the assumed 'pure' populations of the two species in this study). These barcoding sites were then assessed across all samples from the Van Stadens Wildflower Reserve for the presence (or absence) of 2ISPs indicative of hybridisation. The cpDNA *3'trnV*$^{(UAC)}$ region was also sequenced for these samples to determine the maternal origins of each hybrid individual. PCR and sequencing procedures are described in Appendix A.

To assess morphological variation, length and width of five leaves from each plant were measured; these were averaged per individual and converted into a length to width ratio to compare with ITS DNA barcoding sites. In addition, *P. susannae* leaves exude a distinctive odour of sulphur when crushed (by hand) and this is absent in *P. eximia* (Rebelo, 2001). These observations are useful in identifying potential hybrids in the field during sampling. We considered the leaf-odour as an independent trait for assessing introgression of *P. susannae* into individuals that morphologically were more *P. eximia*-like.

2.3) Results

Protea eximia has more oblong leaves (60–110 mm long and 30–65 mm wide) whereas *P. susannae* has more lanceolate leaves (80–160 mm long and 15–30 mm wide).The ITS alignment consisted of 647 base pairs with 26 variable sites, of which 14 were parsimony informative. Of these, only one site (bp 234 (ITS2: 15)) was found to be a reliable barcode for both species — this site also contained the expected 2ISPs indicative of hybridisation in some of the samples from the Van Stadens Wildflower Reserve. Other sites were either autapormophies or were already polymorphic in the parent species (Table 2.1). The

barcoding site was a Cytosine (C) for *P. eximia* and Adenine (A) for *P. susannae* (Table 2-1). Of the 24 samples sequenced from the reserve, 11 samples contained the 2ISP base code ("M") indicative of a hybrid. Plants identified as pure *P. susannae* from the ITS barcoding site had high leaf length to width ratios (>3), whereas plants identified as *P. eximia* had low leaf length to width ratios (<3). However, hybrid individuals had leaf length to width ratios that spanned the range of both *P. eximia* and *P. susannae* (Table 2.1). Note that two hybrid plants with leaf length to width ratios within or near the range of *P. eximia* did not have leaves that smelled like sulphur when crushed in the field. One hybrid individual with a *P. eximia*-like leaf length to width ratio (i.e. < 3) contained the sulphur odour (Table 2.1). The cpDNA alignment consisted of 478 base pairs with 17 variable sites, of which 13 were parsimony informative. Of these, only two sites (a transversion [C→A] and an indel) were observed to have species-specific affinity and thus reliably show the maternal gene inheritance for each individual (Table 2.1).

Table 2.1(next page): Summary of the: i) DNA sequence data from the internal transcribed spacers of the ribosomal cistron (Genbank accessions: MH016411-MH016459), ii) leaf dimensions with a colour gradient of L: W ratios (green=low values; red=high values), iii) the presence of a sulphurous smell in the leaves and iv) summary of the DNA sequence data from the *3'trnV*$^{(UAC)}$ noncoding chloroplast region for *Protea* species (Genbank accessions: MH024397-MH024445) . Base 234 (ITS2: 15) of ITS was used to barcode the two species and identify hybrids. Bases that were not sequenced in certain samples are represented by ? Localities: GNR= Garcia Nature Reserve, LS= Lady's Slipper, VSWFR= Van Stadens Wild Flower Reserve, GM= Gouritzmond, CAG= Cape Agulhas.- represents the sequence of indels: - - - - - - - ; * represents the sequence of ATAAAAA.

					ITS1																						5.8S			ITS2						ndhC-trnV		Leaf Dimensions (mm)			Sulphur
					32	47	65	66	72	76	103	113	122	133	149	155	189	192	203	211	213		1	2	124		15	158	193	203	207	234		56	102				
				Consensus	C	C	G	A	C	A	C	C	C	C	C	T	C	T	C	C	C		C	G	C		C	G	G	T	T	C		G	*				odour
Species	Location	LAT (S)	LONG (E)	Sample ID																																	Length	Width	L:W	
P. eximia	GNR	33.9530	21.2153	PRA124	.	.	.	.	.	.	.	.	.	.	.	C	Y	C	.	.	T		.	.	.		.	.	.	C	C	.		.	—				×
P. eximia	GNR	33.9530	21.2153	PRA125	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		.	.	.	.	.	.		.	—				×
P. eximia	GNR	33.9530	21.2153	PRA126	.	.	K	R	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		.	.	.	.	.	.		.	—				×
P. eximia	LS	33.8826	25.2481	PRA127	.	S	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.		.	.	.		.	.	R	.	.	.		.	—				×
P. eximia	LS	33.8826	25.2481	PRA131	~	S	K	.	.	R	.	.	.	.	.	.	.	.	.	.	.		.	.	.		.	.	.	.	.	.		.	—				×
P. eximia	LS	33.8826	25.2481	PRA128	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		.	.	.	.	.	.		.	—				×
P. eximia	LS	33.8826	25.2481	PRA130	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		.	.	.	.	.	.		.	—				×
P. eximia	LS	33.8826	25.2481	PRA129	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M	.	.		.	.	.		.	.	.	.	.	.		.	—				×
P. eximia	LS	33.8826	25.2481	PRA132	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M	.	.		.	.	.		.	.	.	.	.	Y		.	—				×
P. eximia	VSWFR	33.9087	25.2056	PRA009	.	.	K	R	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		.	.	.	.	.	.		.	—				×
P. eximia	VSWFR	33.9078	25.2101	PRA008	.	.	K	R	.	.	M	.	Y	Y	.	.	.	.	.	Y	.		.	.	.		.	.	.	.	.	.		.	—				×
P. eximia	VSWFR	33.9089	25.2056	PRA011	~	.	.	.	.	R	.	.	Y	Y	.	.	.	.	.	Y	.		.	.	.		.	.	.	.	.	.		.	—				×
P. eximia	VSWFR	33.9093	25.2062	PRA014	~	S	K	.	.	R	.	.	.	.	.	.	.	.	.	.	.		.	.	.		.	.	.	.	.	.		.	—				×
P. eximia	VSWFR	33.9089	25.2064	PRA015	.	.	.	.	.	.	.	.	Y	Y	.	.	.	.	.	Y	.		.	.	.		.	.	.	.	.	.		.	—				×
P. eximia	VSWFR	33.9094	25.2064	PRA016	~	~	~	~	~	~	.	.	.	.	.	.	.	.	.	.	.		.	.	.		.	.	.	.	.	.		.	—				×
P. eximia	VSWFR	33.9086	25.2064	PRA018	.	.	K	R	.	.	.	.	.	Y	.	.	.	.	.	.	.		.	.	.		.	.	.	.	.	.		.	—				×
P. eximia	VSWFR	33.9082	25.2063	PRA020	.	.	K	R	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		.	.	.	.	.	.		.	—				×
P. eximia	VSWFR	33.9082	25.2063	PRA021	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.	Y		.	.	.	.	.	.		.	—				×
P. eximia	VSWFR	33.9041	25.2132	PRA028	~	~	~	~	~	R	.	.	.	.	G	.	.	.	.	.	.		.	.	.		.	.	.	.	.	.		.	—				×
Hybrid	VSWFR	33.9019	25.2106	PRA035	.	.	.	.	Y	.	.	.	Y	Y	.	.	.	.	.	Y	.		.	.	.		M	.	.	.	.	.		.	—				✓
Hybrid	VSWFR	33.9092	25.2189	PRA029	.	.	K	R	Y	.	.	.	Y	Y	.	.	.	.	.	Y	.		.	.	.		M	.	.	.	.	.		T	*				×
Hybrid	VSWFR	33.9042	25.2131	PRA027	~	S	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		M	.	.	.	.	.		T	*				✓
Hybrid	VSWFR	33.9019	25.2103	PRA034	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		M	.	.	.	.	.		T	*				×
Hybrid	VSWFR	33.9034	25.2146	PRA031	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	.		.	.	.		M	.	.	.	.	.		T	*				✓
Hybrid	VSWFR	33.9092	25.2059	PRA012	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		M	.	.	.	.	.		.	—				✓
Hybrid	VSWFR	33.9092	25.2059	PRA013	.	.	.	.	Y	.	M	.	.	.	.	.	.	.	.	.	.		.	.	.		M	.	.	.	.	.		.	—				✓
Hybrid	VSWFR	33.9044	25.2132	PRA024	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M	.	.		.	.	.		M	.	.	.	.	.		T	*				✓
Hybrid	VSWFR	33.9064	25.2097	PRA023	A	.	K	R	Y	R	.	.	.	.	S	.	.	.	.	.	.		.	.	.		M	.	.	.	.	.		.	—				✓
Hybrid	VSWFR	33.9042	25.2131	PRA026	.	.	.	.	Y	.	.	.	.	.	S	.	.	.	.	.	.		.	.	.		M	.	.	.	.	.		.	—				✓
Hybrid	VSWFR	33.9033	25.2146	PRA030	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		M	.	.	.	.	.							✓
P. susannae	VSWFR	~	~	PRA001	.	.	.	.	Y	.	.	.	.	.	.	.	Y	.	.	.	.		.	.	.		A	.	.	.	.	.		T	*				✓
P. susannae	VSWFR	33.9034	25.2146	PRA032	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		A	.	.	.	.	.		T	*				✓
P. susannae	VSWFR	33.9023	25.2111	PRA036	~	S	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		A	.	.	.	.	.		T	*				✓
P. susannae	GM	34.2546	21.7164	PRA097	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.		.	R	.		A	.	.	.	.	.		T	*				✓
P. susannae	GM	34.2546	21.7164	PRA098	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		A	.	.	.	.	.		T	*				✓
P. susannae	GM	34.2546	21.7164	PRA099	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		A	R	.	.	.	.		T	*				✓
P. susannae	GM	34.2546	21.7164	PRA100	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		A	.	.	.	.	.		T	*				✓
P. susannae	GM	34.2546	21.7164	PRA102	.	.	.	.	Y	.	.	.	.	.	.	.	Y	.	.	.	.		.	.	.		A	.	.	.	.	.		T	*				✓
P. susannae	GM	34.2546	21.7164	PRA104	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		A	.	.	.	.	.		T	*				✓
P. susannae	GM	34.2546	21.7164	PRA106	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		A	.	.	.	.	.		T	*				✓
P. susannae	CAG	34.5856	19.8184	PRA107	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		A	.	.	.	.	.		T	*				✓
P. susannae	CAG	34.5856	19.8184	PRA111	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		A	.	.	.	.	.		T	*				✓
P. susannae	CAG	34.5856	19.8184	PRA110	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		A	.	.	.	.	.		T	*				✓
P. susannae	CAG	34.5856	19.8184	PRA108	.	.	.	.	Y	.	Y	.	.	.	.	.	.	.	.	.	.		.	.	.		A	.	.	.	.	.		T	*				✓
P. susannae	CAG	34.5856	19.8184	PRA109	.	.	.	.	Y	.	Y	.	.	.	.	.	.	.	.	.	.		.	.	.		A	.	.	.	.	.		T	*				✓
P. susannae	CAG	34.5856	19.8184	PRA114	.	.	.	.	T	.	Y	.	.	.	.	.	.	.	.	.	.		Y	.	.		A	.	.	.	.	.		T	*				✓
P. susannae	GM	34.2546	21.7164	PRA103	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.		Y	.	.		A	.	.	.	.	.		T	*				✓
P. susannae	CAG	34.5856	19.8184	PRA112	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.		Y	.	.		A	.	.	.	.	.		T	*				✓
P. susannae	CAG	34.5856	19.8184	PRA113	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.		A	.	.	.	.	.		T	*				✓

~: missing data; *: ATAAAAA; —:gaps

2.4) Discussion

Despite numerous field observations of hybridisation in the genus *Protea* recorded by the Protea Atlas Project observers more than a decade ago, these results are the first to provide unequivocal evidence of uncontrolled (i.e. not specifically bred) hybridisation between a local and introduced species in the genus. This lack of investigation into the genetic (and ecological) consequences of translocations is a general problem for Cape lineages (highlighted in Potts, 2017).

The number of hybrids detected using ITS was higher than expected from field observations of the leaf and floral morphology. A few ITS-identified hybrids were strongly *P. eximia*-like or *P. susannae*-like in their morphology and odour. Although some hybrids did have morphological cues, such as intermediate (e.g. in leaf size and shape) or mixed (e.g. *P. eximia*-like leaves with a sulphurous odour) phenotypes, the strongly parent-like hybrids would have been undetected in morphological analyses; this would lead to an underestimation of the extent of hybridisation. In Australia, strong morphological differentiation was maintained despite hybridisation between sympatric species of *Lomatia* R.Br. (Proteaceae; McIntosh et al. 2014), which further highlights the importance of genetic analyses to identify hybrids. Floral morphology was detected in-field as a potential indicator of hybrids in some instances (e.g. Fig. 2.3); however, plans to assess this were ruined by the non-demonic intrusion (Hurlbert, 1984) of the wildfire in June 2017 — all flower heads were destroyed.

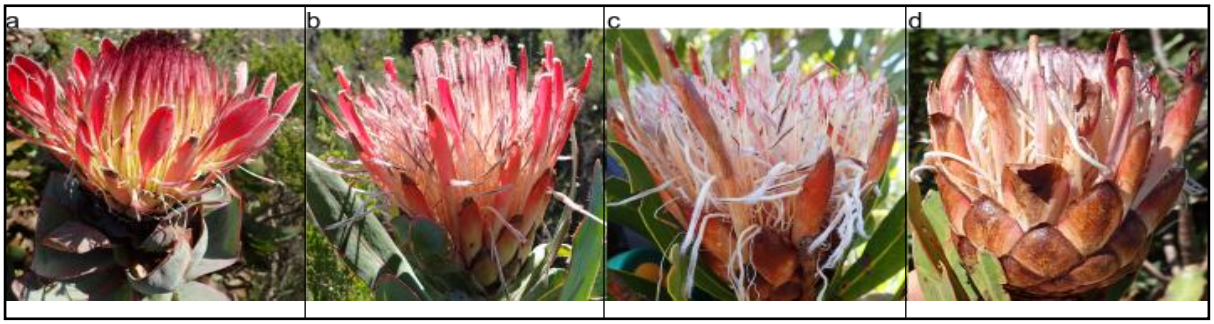


Fig. 2.3. Changes in floral morphology between (a) *P. eximia* (PRA011), (b) *P. eximia*-like hybrid (PRA012), (c) *P. susannae*-like hybrid (PRA031) and (d) *P. susannae* (PRA036).

Hybrids were found across the entire sampling range within the reserve where *P. eximia* and/or *P. susannae* were found (Fig 2.2). Hybrid plants contained maternally inherited cpDNA from both *P. susannae* and *P. eximia*. (Table 2.2) — this demonstrates that pollen flow has been bi-directional across these species. More in-depth sampling of the next generation may help quantify the distance of gene flow via pollen versus seeds. Sampling further afield will be required to determine the edge of the hybridisation front and to estimate the rate of spread.

Table 2.2: Comparison of nuclear and chloroplast signals.

		nDNA (ITS)		
		Protea eximia	Hybrids	*Protea susannae*
cpDNA (*trnV*$^{(UAC)}$)	*Protea eximia*	19	6	0
	Protea susannae	0	5	19

This study demonstrates that hybridisation between local and non-local species poses a threat to species genetic integrity at the population level (*sensu* Laikre et al., 2010). Could this be the case for other Cape lineages? As the Cape has a long evolutionary history of climatic and topographic stability (Hopper, 2009; Cowling et al., 2015), lineages are likely to have allopatrically speciated without subsequent range shifts (normally driven by shifting climate) and so closely-related species rarely came into contact — thus reproductive isolating mechanisms are unlikely to have evolved. In contrast, plant species in the northern hemisphere (e.g. Europe and North America) experienced dramatic climate variation during the Pleistocene; thus range shifts were common and reproductive barriers were required to maintain species identities. Glacial expansions led to dramatic southward migrations of northern hemisphere plants (Rowe et al. 2004) driving increased inter-species encounters. Plants likely developed adaptations to prevent genetic mixing in these potential 'hybrid zones' (Hewitt, 1996).

In the Cape, where many local species are geographically restricted and have small population sizes, hybridisation with an introduced non-local species may severely compromise its genetic integrity (i.e. ‘genetic swamping’, reviewed in Laikre et al. 2010). An unexplored example is another member of the Proteaceae in the Van Stadens Wildflower Reserve: the range-restricted and endangered population of *Leucadendron orientale* I.Williams is potentially under threat due to suspected hybridisation with the introduced and non-local *Leucadendron tinctum* I.Williams (G. Matsha, reserve manager, pers. comm.).

Members of the *Protea* genus are key cultivars in the South African cut flower industry (Middelmann, 2012) — in this industry hybrids are actively crossed (Coetzee and Littlejohn, 2007) and species have been commonly translocated to increases the diversity of available flowers. For example, *P. susannae* is the parent species to numerous hybrid crosses such as ‘Pink Ice', 'Special Pink Ice', 'Cardinal', 'Sylvia' and 'Susara' (Williams et al., 2008; Gerber et al., 2001). ‘Protea Sylvia’ in particular is sought out for cultivation. As a result, many *Protea* species (and other members of the Proteaceae) have been introduced into new areas alongside native populations of other species (Reinten et al., 2011). Thus, hybridisation and genetic swamping may be far more common than currently evident in the scientific literature — it has simply not been studied and is difficult to readily study infield as comparison with parents is often not possible in a post-fire generation. This study provides the first genetic evidence of “Frankenflora” (hybridisation resulting from species translocations), and I hope this will stimulate further research that ultimately will quantify the potential threat that intra-Cape lineage hybridisation poses to the genetic-component of biodiversity. The ecological threat of hybrids due to hybrid vigour also requires urgent attention — i.e. hybrids that behave as invasive species.

Chapter 3: Detecting hybrids: exploring low cost and rapid molecular methods

3.1) Introduction

There are many methods available to identify hybrids, such as morphology, cytology, secondary chemistry and molecular markers (López-Caamal and Tovar-Sánchez, 2014). Unsurprisingly, there has been an increase in the use of molecular markers over more traditional methods, such as morphology, to study patterns of hybridisation (e.g. Twyford and Ennos 2012). There are two branches of molecular ecology: Phylogenetics and Population genetics. Phylogenetics usually detects more ancient hybridisation (but also occasionally recent *sensu* Gerard et al., 2011; Yu et al., 2013). However, hybridisation is difficult to explicitly test and is usually inferred. Population genetics focuses more on individuals and usually contains a spatial component. The methods used in both branches are often quite different. For example Amplified fragment length polymorphisms (AFLP's) and Microsatellites are used at the population genetics level, whereas DNA sequencing is commonly used in phylogenetics and is also used in population genetics but only when variation is high (however, this is rarely the case in plants). Nevertheless, in the era of Next Generation Sequencing (NGS) these two fields share a common approach. NGS provides sufficient data for both population genetics and phylogenetics. An example of this is the use of a Radseq (NGS) approach by Mitchell and Holsinger (2018), who used Radseq data to discriminate between *Protea puncata and Protea venusta* and their putative hybrids but also the same type of data were used to develop a phylogeny for *Protea* L. (Mitchell et al., 2017).

Here I use an unusual method to detect hybrids based on an identified Single Nucleotide Polymorphism (SNP) ,identified in Chapter 2(Site ITS2 15 Table 2.1 pg. 32) that we can differentiate using PCR methods. Sections 3.1.1 to 3.1.6) provide a review of the methods commonly used thus far to place these new methods in context in context with the PCR methods. These two new methods are species-specific primers and High Resolution Melt (HRM) respectively.

Methods to identify hybrids

In chapter 2, Sanger sequencing was used to detect hybrids produced between *Protea eximia* (Salisb. ex Knight) Fourc. and *Protea susannae* E.Phillips at the Van Stadens Wildflower Reserve. This was an effective approach, however, to develop a geographic distribution of hybridisation would require hundreds to thousands of individuals to be sequenced — this would be prohibitively expensive. For in-depth study of this system, inexpensive, alternative methods of detecting hybridisation are required; so what are the options? Here I explore two alternative molecular methods to Sanger sequencing, namely High Resolution Melt analysis (HRM) and a Species-Specific Primer Polymerase Chain Reaction (PCR) based approach to hybrid identification.

3.1.1) Sanger Sequencing (Table 3.1)

First generation sequencing (also known as Sanger sequencing) has been regarded as the gold standard for nucleic acid sequencing for the last two and a half decades (Grada and Weinbrecht, 2013). It is still used as the benchmark to which Next Generation sequencing is compared today (Sanger et al., 1977; Grada and Weinbrecht, 2013). Sanger sequencing produces a highly accurate nucleotide sequence with relatively long reads (>700bp). The main downside to Sanger sequencing is the cost per sample. Despite falling costs, it is still prohibitively expensive for use in large molecular ecology studies (Hudson, 2008). The costs of Sanger sequencing are unlikely to come down sufficiently (Holt and Jones, 2008).Thus, alternatives to Sanger Sequencing are required. These alternatives vary in option and cost. The options include PCR based methods such as HRM and microsatellites and Next Generation Sequencing methods (Do Amaral et al., 2015). The Next Generation sequences, however, can be even more expensive per sample.

3.1.2) Microsatellites (Table 3.1)

Microsatellite markers, simple sequence repeats (SSRs, Jacob et al., 1991) or short tandem repeats (STR's, Edwards et al., 1991) are tandem repeats of one to six nucleotide long DNA motifs (Kalia et al, 2011). Such repeats and length variation of repeats are found in in all prokaryotic and eukaryotic organisms analysed to date (Zane et al., 2002). Microsatellites sample widely across the genome as opposed to the PCR approach for Sanger sequencing which samples a specific small part of the genome. They have emerged as a popular and versatile marker choice for ecological applications (Selkoe and Toonen, 2006). This is due to their many positive genetic attributes accredited to them, specifically hypervariability, multialleic nature, extensive genome coverage of organelle genomes and high quantity genotyping (Kalia et al., 2011; Gémes Juhász et al., 2006). The extremely high polymorphism of microsatellites has revolutionised the genetic identification of individuals — this is known as "DNA fingerprinting" (Gill et al., 1985). Microsatellites can be developed from genomic DNA libraries or can be found on public databases such as GenBank as early as 1984. Tautz and Renz (1984) confirmed the abundance of microsatellites in plants. Plants are very rich in AT repeats. Microsatellites are also useful in the detection of hybridisation. The highly variable, co-dominant nature of microsatellites allows the allelic contribution of each parent to be detected. Their rapid rate of mutation allows for differentiation between closely related species and species-specific alleles can be used to estimate levels of interspecific gene flow (Powell et al., 1996; Muir and Schloetterer, 2005).

Originally microsatellites were used in studies of plant pollination, seed dispersal (Chase et al., 1996; Dow and Ashley, 1998, Streiff et al., 1999) and studies assessing population genetic structure within a species (Frair et al., 2001). More recently they have been used in studies on plant hybridisation (e.g. Glaubitz et al., 2001; Craft and Ashley, 2007; Scascitelli et al., 2010; Gauli et al., 2014; Zaya et al., 2015). Craft et al. (2002) used microsatellite data to estimate the hybridisation frequency between *Quercus lobata* Née and *Quercus douglasil* Hook. & Arn.. The high level of differentiation between the microsatellite loci in the species suggested that these markers may provide improved resolution for studying hybridisation in oaks. Their results of their study showed clear introgression between the two species, but that this was a rare occurrence and there does appear to be a strong fertility barrier between *Q. lobata* and *Q. douglasii*.

There are notable disadvantages to using microsatellites to study of hybridisation. First, a DNA library for the model taxa (parent species or genus) in question needs to be created and screened for microsatellites (Dow and Ashley, 1996). Then the regions flanking the loci must be sequenced and then finally PCR primers must be designed for these loci. It is a long and complicated process. Substantial financial and time costs are required to build up the genomic library before microsatellite primers can be designed (Ashley and Dow, 1994). The second is the steep learning curve required for researchers before they attempt microsatellites (Selkoe and Toonen, 2006). Thirdly is that the use of microsatellites is restricted to closely-related hybridising species. Primers designed for one species may not be widely applicable for use across the genus when determining parental origins of a hybrid taxon (Sun and Lo., 2011).

3.1.3) RAPD Markers (Table 3.1)

Another popular method that can be effectively used to detect hybrids are Random Amplification of Polymorphic DNA (RAPD) markers. Baird et al. (1992) used RAPD markers to identify inter-specific and intra-specific hybrids between *Solanum* L. species. This method is technically simple to replicate and only requires small quantities of DNA (Wilde et al., 1992). At the individual level, RAPD markers may be applied to determine parentage and identify hybrids (Hadrys et al., 1992). Arnold et al. (1991) demonstrated gene flow between two Louisiana hybrid species, *Iris fulva* Ker Gawl. and *I. hexagona* Walter, using species-specific RAPD markers. Markers specific to *I. fulva* were not present in *I. hexagona*. However, these same markers were present in experimentally produced F1 hybrids (*I. fulva* x *I. hexagona*).

However, the reproducibility of RAPD data has come into question (Sunnucks, 2000). At present, it is no longer accepted for population level studies in leading journals (for example, see: *Molecular Ecology* Guide to Authors, 2018). RAPD has also been primarily criticised due to competitive priming (Weising et al., 1995). It would seem, therefore, that RAPD markers would not be a good choice to test for hybridisation between species in the modern genetic era.

3.1.4) High resolution Melting (HRM) (Table 3.1)

High-resolution melting (HRM) analysis is a closed-tube method for rapid analysis of genetic variation within PCR amplicons (Reed and Wittwer, 2004). Upon completion of PCR in the presence of a saturating intercalating dye (which binds to double-stranded, but not single-stranded DNA) the PCR product is heated, while the level of fluorescence is measured. As the temperature rises and the duplex melts, dye is released, and fluorescence intensity is reduced. Genetic variants with differences in base composition result in differences in melting temperature, which are detected by monitoring fluorescence during an increase in temperature and discriminated by their characteristic melting curves. This is visualized by a loss of fluorescence as the DNA duplex melts (Zhou et al., 2005). Before the HRM takes place, primers need to be designed to flank the gene region to be amplified. Prior knowledge of the sequence variation is required to know where to place the primers for an optimal result. A curve is produced for each specific sequence (Simko, 2016). What results is a low-cost tool that can be used to quickly screen for variations amongst PCR products (Wittwer, 2009; Hofinger et al. 2009). Considered the next generation of amplicon melting analysis (Garritano et al., 2009), HRM can be used to detect single base pair differences (up to 100 bp under optimal conditions) (Malentacchi et al., 2009). Wittwer (2009) however, states that some heterozygotes (individual with different alleles at a given locus) with observed as different while still differing from other homozygotes (individual with the same alleles at the locus), may produce melting curves so similar that they are difficult to differentiate between. The safest option in this case is to test samples prior to HRM analysis using software such as uMelt Batch 2.0 (Dwight et al., 2011). This tells us that without properly preparing of the primers, the HRM curves are difficult to differentiate between.

Ganopoulos et al. (2013) developed a method of identifying hybrids in the *Pinus* genus utilising the HRM technology. Chloroplast DNA (cpDNA) inheritance, via pollen transfer, occurs in gymnosperms (Palmer et al., 1988). Introgression between *Pinus halepensis* Miller and *Pinus brutia* Tenore produced F_1 offspring in a glasshouse. The method first amplified the *trnL cpDNA* regions to identify the different species of *Pinus.* HRM analysis was then

used to discriminate between different DNA sequences of *P. brutia*, *P. halepensis* and their F1 hybrid offspring. The melting curves produced using the HRM software were sufficiently dissimilar that the two species and their hybrid offspring could be readily discriminated. The melting curves showed that all the hybrids claimed paternal inheritance from *P. halepensis,* with the melt curves positioned closer to the pure *P. halepensis.* It could produce species-species comparisons to allow for an explicit proof of hybridisation between *P. halepensis* and *P. brutia* in orchards. The problem with this study was that it made use of hybrids anthropogenically cultivated in a glasshouse. Inheritance and the gene flow of hybrids is more difficult to determine in cases of natural hybridisation that has occurred over multiple generations. Backcrossing and introgression with pure parental species may also occur (Nolte and Tautz, 2010), which can complicate determining the maternal or paternal inheritance of individuals.

HRM has also been used to genotype cultivated plants. For example, Distefano et al. (2015) used HRM and SNP markers to successfully genotype nine citrus species and one hybrid cultivar. HRM was shown, in this example, to successfully distinguish each citrus species from hybrids. HRM is also cost and labour efficient; for example only one technician is required to run the machine for each two-hour screen of 96 samples (Ujino-Ihara et al., 2010). HRM analysis is highly robust and highly sensitive to single changes in base-pairs between species (Rodríguez López et al., 2010).

Medford (2016) also found that HRM successfully detected hybrids forming between *Castilleja mollis* Pennell and *Castilleja affinis* Hook. & Arn.. She used HRM because it offered a more streamlined and affordable technique to process many samples over a short period of time. In her results she found that some of the HRM curves accurately differentiated the two known parent species from one another. However, when unknown samples were added it was not possible to distinguish between the two species and the hybrids. Thus, there were no conclusive results for the hybrids, so more work needs to be done on the HRM front to show that *C. mollis* and *C. affinis* can be differentiated from their hybrid offspring

3.1.5) Species-specific primers and PCR (Table 3.1)

PCR is a standard tool to amplify gene regions and forms the basis of most DNA methods. Primers designed specifically to flank species specific Single Nucleotide Polymorphism (SNPs) have been used widely in the past (Gupta et al., 2001; Vignal et al., 2002; Sweeny et al., 2007). The internal transcribed spacer (ITS) is a region between the 18 and 26S nuclear RNA (Baldwin, 1992) and is very popular for use in plant barcoding as it is flanked by

conserved regions for designing universal primers, is a multicopy and subject to recombination (Li et al., 2011; Vijiyan and Tsou, 2010).

The species-specific primer approach developed in this chapter was inspired by Chapman et al. (2002) — who utilized known autapormorphies in the ITS2 nuclear and mitochondrial cytochrome *b* regions across shark species to specifically detect white shark DNA out of samples of unknown shark species origin. To address the problem of illegal harvesting of great white sharks they developed a rapid, molecular assay, species-specific PCR primer designed for accurate identification of white shark body parts. It is a very sensitive tool that can detect white shark DNA in a mixture of up to 10 commercially fished shark species pooled in a single PCR tube.

I used an autapomorphic SNP (at least for one species) that differentiates the two species. The SNP has been identified in the ITS2 region in Chapter 2 (Table 2.1). Each species requires a unique fixed substitution i.e. all individuals of the same species share the same base and that base is different from other species. This is difficult in plants as there may be intra-species variation (i.e. not fixed) or within a species a substitution may be shared with other species. Thus, this approach will only work if there is a fixed substitution of bases between species. DNA barcoding tries to use such fixed species substitutions to identify species (Ekblom and Galindo, 2011). In animals (such as sharks) it works well but it does not work so well for plants. There are many barcoding plant papers that show that ITS fails to differentiate amongst species (e.g. Cowan et al., 2006; Rubinoff et al., 2006; Fazekas et al, 2009). The use of cpDNA (chloroplast DNA) barcoding does not offer much in terms of identifying hybrids. This is because cpDNA can only provide information on maternal inheritance. It does not provide a SNP that discriminates species and hybrids from each other (Taberlet et al., 2006). Species-specific primers will only work for certain model systems where an autapomorphy is present. So, a bit of luck is required to apply this approach to a given model system.

3.1.6) Next Generation Sequencing (NGS) (Table 3.1)

The power of NGS lies in the vast quantity of reads it can produce. A typical NGS can yield hundreds of millions of short reads per run. This is as opposed to Sanger sequencing, which can only yield a few hundred bases (Metzker, 2010). NGS technologies generate a large quantity of complex nucleotide sequence data from populations. By allowing for new genomic tools to be generated for organisms that have no prior sequence data, NGS

promises to dramatically improve our ability to study hybridisation and introgression (Hohenlohe et al., 2011). As stated earlier, NGS sequencing technologies bridges the divide between population genetics and molecular systematics.

Although many studies for hybrid testing have been performed using NGS on animals, the number of NGS studies done on plants is limited (Jain, 2011). There are only four known studies, that have use NGS to detect hybridisation in plants. Lai et al. (2012) produced 22 Sanger Expressed Sequence Tags (ESTs) from 11 weeds in the sunflower family using Sanger, Roche 454 and Illumina sequencing. Roche 454 sequencing is a directed sequencing that can meet 20,000+ reads of up to 700 bp. The greatest advantage of Roche 454 sequencing is its speed. It takes only 10 hours from sequencing start to completion (Liu et al., 2012). One of its shortcomings is its high error rate. Illumina sequencing produces many high precision sequencing reads. It has a much lower error rate than Roche 454 sequencing (less than 2%). One disadvantage of Illumina sequencing is that it is generally not able to assemble the chloroplast genome of plants (Wang et al., 2018). Ks values (number of synonymous substitutions per synonymous site) were produced by the Roche 454 and Ilumina sequencers, respectively; Ks values are the result of a Kolmogorov–Smirnov (Ks) test, which is a non-parametric method for comparing two samples. For some taxa the distributions of Ks values were compared between orthologs of congeneric taxa to detect hybridisation (Kane et al., 2009). The distribution of the Ks values for orthologs should be centred around a Ks value corresponding to the time since the last common ancestor of the taxa involved. However, a secondary peak at a lower Ks value can form, which can be accredited to more recent gene flow (Lai et al., 2012). Thus, this is used as an indicator of hybridisation.

Another NGS hybrid detection example, already mentioned briefly in is the study by Mitchell and Holsinger (2018), who utilised Restriction Site Associated (RSA) genotyping by Sequence (RadSeq) to identify hybrids between two closely related and morphologically similar species of *Protea*. RadSeq is a targeted next generation sequencing approach. It is a high-throughput technique based on restriction site associated DNA sequencing that enables the genotyping of thousands of genetic markers for any species (Andrews et al., 2016). This includes non-model organisms. RAD-tags (the DNA markers that flank the RADSeq data) were used to identify unique SNP's for the identification of *Protea puncata* Meisn. *and Protea venusta* Compton*,* along with an intermediate form which would indicate hybridisation. The RADSeq data were, therefore, able to reveal extensive hybridisation between these two species in the wild.

Eaton et al. (2015) utilised RADSeq data to identify hybrids in oak species. They determined that other methods, such as ABBA-BABA tests, to identify introgression of the genomes of extant species had been widely used. However, these methods are integrally comparative and are sensitive to the effects of missing data. By utilising genomic RADSeq data sampled from all extant American oak species (*Quercus* spp.), a group notorious for hybridisation, they were able to distinguish true hybridising lineages from those that falsely appeared as an admixture. Six of seven species showed evidence of hybridisation, often with many other species. This can be explained by introgression of a few lineages living in close proximity. The RADSeq data showed that introgression among oaks to be highly localized. This makes sense as oak species boundaries and geographic ranges have remained stable over geological periods (Anderson and Ferree, 2010).

A classic example of hybridisation must be the Californian sunflowers (Owens et al., 2016). *Helianthus bolanderi* A.Gray is thought to be a hybrid of the endemic species, *Helianthus exilis* A.Gray and the invader, *Helianthus annuus* L. The NGS approach of genotyping by sequencing was used to look for evidence of introgression and population structure. They found that *H. bolanderi* and *H. exilis* form one generic clade and a single species, not two. While their results failed to support the hybrid origin of *H. bolanderi*, it did detect some evidence of hybridisation into the invader *H. annuus*.

NGS is very useful as it can sequence the entire genome or large parts of it.(Davey et al., 2011). It is beneficial for large scale hybridisation projects where it can determine the spatio-temporal dynamics of hybrid zones, determine the significance of reticulate evolution in species formation and ascertain the behaviour of introgressed loci in their new genomic background (Twyford and Ennos, 2012). When introgression between species in the wild is being investigated, NGS can be employed to generate an array of informative molecular markers between closely related species. An example of this is *Populus* L. where SNP's have been assayed for 635 individuals (Thompson et al., 2009). It is advantageous to use NGS technology in cases where there are many samples to sequence. However, the extreme cost is a dissuading factor for its use in small scale hybridisation identification projects (Koboldt et al., 2013). For projects with smaller numbers of samples (10-50 samples) the benefits of using of NGS is outweighed by the cost. Species-specific primer for PCR-identification can be generated after barcoding sites have been identified from NGS studies. Thus, the species-specific primer approach used in this chapter is still relevant in a NGS era.

Table 3.1: Summary of the advantages, disadvantages, research discipline and referenced of the different markers used for hybrid identification

Marker	Marker type	Advantages	Disadvantages	Field	Reference
DNA sequencing (Sanger)	Locus specific	Universal primers	Expensive to apply, limited nuclear regions available. Low information in plants	Phylogenetics and sometimes population genetics	Grada and Weinbrecht (2013)
NGS	Targets whole genome	Many reads for little effort	Expensive and steep learning curve High error rates	Phylogenetics and population genetics	Metzker (2010)
Amplified Fragment Length Polymorphisms (AFLP)	Whole genome fingerprint	Quick to optimise, Many loci, cheap, fast	Reproducibility issues	Population genetics	Simpson (1997)
Microsatellites (Simple Sequence Repeat Markers (SSRs))	Short tandem repeats	Polymorphic, co-dominant	Hard to automate. SSRs are often species-specific	Population genetics	Kalia et al (2011)
RAPD markers	Type of PCR. DNA segments amplified are random	Simple to replicate. Only requires small amounts of DNA	Reproducibility issues. Competitive priming	Population genetics	Baird et al. (1992)
High Resolution Melt (HRM) PCR	Duplex specific	High sensitivity. Less time consuming than other methods (i.e. immediate results post-PCR)	Difficult to tell heterozygotes apart on melt curves	Population genetics	Reed and Wittwer (2004)
Species-specific primers		Cheap and relatively easy to design	Only works with species specific SNP's. Must design a new primer for each new species.	Population genetics	Chapman et al. (2002)

Here I test two PCR-based methods, HRM and species-specific primers, as my model system has one unique nuclear DNA barcoding site for each species identified in Chapter 2. Thus, I corroborate these two methods targeted this section of DNA. Had no discriminating site been available, then a broader genome sampling strategy would have been required,

such as microsatellites or NGS. Both would have been costlier. Microsatellites would have been costlier to design and NGS would have been costlier to run per sample. Here I test methods that only apply to a fixed substitution, but will allow a high throughput of many samples.

3.2) Materials and Methods

3.2.1.) Sampling

The same samples collected for Chapter 2 were used, plus additional, post fire seedlings were collected (for information on the fire see Chapter 2 Materials and Methods); these represent the next generation as all adults in the landscape were killed. Two to three leaves from each seedling were collected semi-randomly to try to get samples of both species from different parts of the landscape. In total 49 individuals were sampled in addition to 24 from the previous generation.

3.2.2) Sanger sequencing

Sanger sequencing is described in Appendix A. In brief, this involved sequencing the ITS1-5.8S-ITS2 nuclear regions and the 3'trnV $^{(UAC)}$ chloroplast region (Shaw et al., 2014)

3.2.3) HRM

The HRM primers were designed to flank the Species-Specific SNP. This was done using the design primer function of *CodonCode Aligner* v6.0.2 (CodonCode Corporation Centerville, Massachusetts). The primers were synthesised by Inqaba Biotech™ (Pretoria). All DNA samples were diluted to 5 ng/µl. Four microliters of DNA were pipetted into a 96 well plate along with a 6 µl mastermix consisting of *Bio-Rad* HRM precision super mix and primer HRM (F1): (GGC GTC ACG CGT ACG TCG CC) and primer HRM (R3): (CGC TCC GCG CTC CGC CGC CA). Each sample was replicated three times to assess melt curve precision. The HRM-PCR was conducted on a *Bio-Rad* CFX Connect™ Real-

Time PCR Detection System. Post-melt analyses were conducted with *Bio-Rad* Precision Melt Analysis v1.2. The amplification cycle consisted of denaturation at 95 °C for 2 min 10 seconds, primer annealing at 60.9 °C for 1 min and primer extension at 72 °C for 30 sec. Samples were subjected to 45 repeats of the above thermal cycle. This was followed by 95 °C for 30 sec., 60 °C for 1 min and 65 °C for 10 sec. The melt analysis consisted of a ramp from 65 °C to 95 °C at 0.2 °C/s for 10 seconds.

A manual filtering procedure was conducted on the HRM-PCR results to eliminate any PCRs that may have failed (This is described further in Appendix C): a log graph of amplification was used to remove any samples that amplified below 10^3 relative fluorescence units (RFU) after 40 cycles (Fig. C1). Next, a melt peak curve was produced and any samples below 1000 d(RFU)/dt (negative derivative of fluorescence vs. temperature) at 90°C was removed (Fig. C1). Finally, a normalized melt curve was produced to compare samples. Samples that had a Normalised RFU of < 0.8 at 87.5 °C were removed (Fig. C1).

The results of the HRM were compared to the results of the Sanger sequencing to see how accurate each method was. The clusters of each HRM run were scored on a *MS Excel* spreadsheet according to true positivity: cluster 1 for a true positive hybrid, cluster 2 for a true positive *P. eximia*, cluster 3 for a true positive *P. susannae* and cluster 4,5,6 etc. for samples that were placed by the *Bio-Rad* Precision Melt Analysis software into an incorrect cluster. An example of a cluster 4 would be a sample sequenced as *P. eximia* by Sanger sequencing, but clustered to *P. susannae* by the CFX HRM machine. A *R*-script was used to determine the specificity and sensitivity of each of the HRM runs (R Core team, 2018; Appendix D).

3.2.4) Species-specific primers (SSP's)

The primers were manually designed so that the 3' end of the primer fell on the (barcoding) species-specific SNP. The number of bases of each primer was increased until their estimated annealing temperatures matched those of ITS4 and ITS5m. Seven primers were designed, three of 18 bp (base pairs) and four of 19 bp. These were named 5' FwdA_19, 5'FwdC_19, 5'FwdA_18, 5'FwdC_18, 5'RevA_19, 5'RevC_19 and 5'RevC_18 (Appendix E, Table E1) after direction, the number of bp and the bp name that they ended in, respectively (Fig 3.1). The intention was to create a multiplex primer that could run two pairs of primers in one PCR. I designed this range of primers to explore which ones functioned optimally.

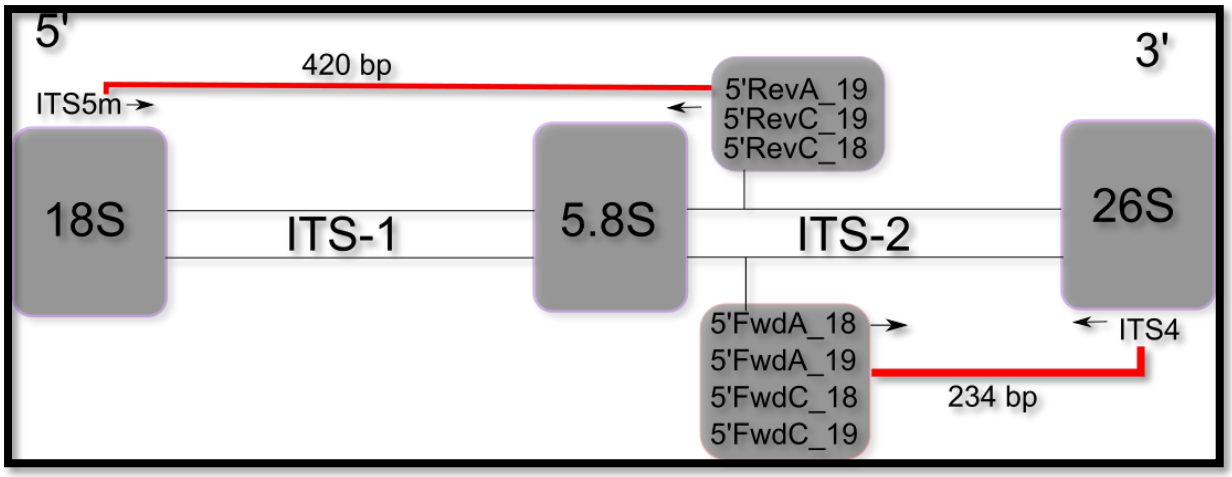


Fig. 3.1 Positions of the primers along the internal transcribed spacer: (C) represents the primers differentiating *P. susannae* and (A) represents the primers differentiating *P. eximia*.

The primers were synthesised by Inqaba Biotech™ (Pretoria). The samples were first tested on a gradient PCR of the Shaw protocol (Shaw et al., 2014). For each run I used two, local *P. eximia*, two non-local *P. susannae* and two hybrid samples. The PCR cycling conditions were template denaturation at 80 °C for 3 min followed by 29 cycles of denaturation at 95 °C for 1 min, primer annealing at a gradient for 1 min, primer extension at 65 °C for 5 min 50 sec and a second annealing at 50°C for 0.50 sec followed by an extension step of 65°C for 4 min followed by a final extension step of 5 min at 65 °C. Samples were then kept at infinite hold at 8 °C. To detect whether PCR amplification was successful the PCR product was checked using electrophoresis on an agar gel at 100 V for 15 min. The gradient annealing temperatures were (50 °C – 66 °C) at increments of 1.2 °C.

I tried the 5'FwdA-ITS4 and 5'FwdC-ITS4 combinations using this protocol but there was no amplification (results not shown). There was successful amplification with the 5'RevC-ITS5m and 5'RevA-ITS5m primers with the Shaw protocol, however there was no discrimination between the *P. eximia* and *P. susannae* i.e. all samples amplified despite the targeting. It was determined that the annealing period in the Shaw protocol was too long (1 min) and was too low. This resulted in non-discriminatory amplification. For example, 10 seconds is considered sufficient time for primers to anneal (Rutland, pers com). I tested the *New England Biolabs* protocol with PCR cycling conditions of: denaturation at 95 °C for 2 min followed by 25 cycles of denaturation at 95 °C for 15 sec , primer annealing at 54 °C for 13 sec (annealing temperature was initially reduced to 10 sec *sensu* Rutland (pers comm) but then it was determined that 13 sec gave stronger amplification) and primer extension at 68 °C for 45 sec followed by a final extension step of 5 min at 68 °C. Samples were then kept at

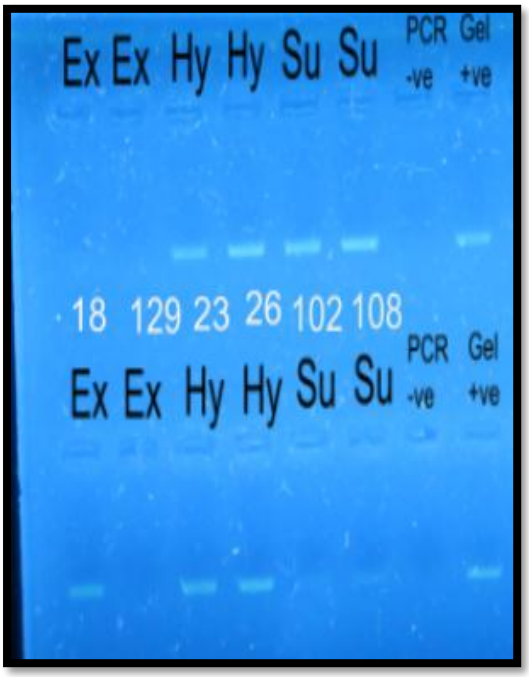
10 °C for an infinite hold. Gradient PCRs were conducted (54 °C to 65 °C at increments of 1.2 °C) and 54 °C was selected as the final annealing temperature.

All samples were amplified in two separate PCRs (5'RevA_19-ITS5m and 5'RevC_19-ITS5m) and the presence or absence of bands was recorded after electrophoresis (1% agar gel at 100 V for 15 min).

If a sample amplified with primers 5'RevC_19 and the ITS5m primers but did not amplify with primers 5' RevA_19 and ITS5m then it was considered to represent a *P. susannae* (Fig 3.2; Table 3.2). If a sample amplified with primers 5'RevA_19 and ITS 5m, but not with 5' RevC_19 then it was determined to be *P. eximia*. If a sample amplified for both primer pairs then it was considered to be a hybrid (Fig 3.2; Table 3.2). If a sample did not amplify with either primer pair it was deemed a failure and re-run.

Table 3.2: Determination of species from gel amplification

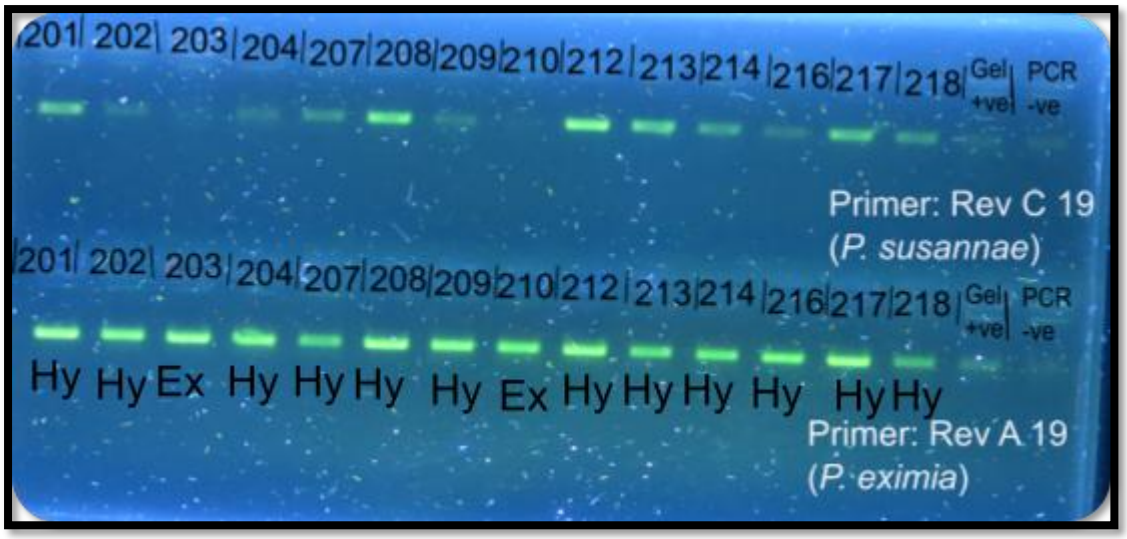
Amplification of:		Result
5'RevC_19 & ITS5m	5'RevA_19 & ITS5m	
X		*P. susannae*
	X	*P. eximia*
X	X	Hybrid



5'RevC_19 *P. susannae*

5'RevA_19 *P. eximia*

A



B

Fig. 3.2 A & B. Gel electrophoresis result for samples subjected to species-specific primers method (Ex = *P. eximia*, Hy = Hybrid, Su = *P. susannae*, Numbers = sample number).

I also conducted a costing analysis to determine the cost of Sanger sequencing, HRM and SSP's for 49 samples each respectively (Appendix F: Table F1-F3).

3.3) Results and Discussion

3.3.1) HRM

The aim of this study was to explore rapid cost-effective methods of hybrid detection. When *Protea eximia* and *Protea susannae* samples collected from outside of this hybrid zone were compared with what were assumed to be F1 hybrids (from the ITS trace files), HRM could clearly discriminate between the three types, i.e. each pure species and the hybrids (Fig. 3.3: A-C). However, when a similar approach was used, but with only samples collected from the hybrid zone and including samples that might be back-crosses (also assumed from trace files), then HRM lost its ability to discriminate hybrids from species (Fig. 3.3: D-E). This is likely because there has been a long history of repeated hybridisiation in this area, and there may be third or fourth generation hybrids that have backcrossed repeatedly with the parental species. Thus, they would have proportionally more copies of one parent species ITS variant

and this would shift the HRM curve towards that of the parent. Thus, I suspect that HRM quickly loses its ability to discriminate past the F1 hybrid event, but this remains to be tested experimentally. HRM can be used with ‘recent’ hybrids, but it must be used with caution. The lack of discrimination is also shown with the decrease in specificity and sensitivity from Fig 3.3: A-C to D-E. This means there were more false positives and false negatives in Fig 3.3: D-E compared to Fig 3.3: A-C.

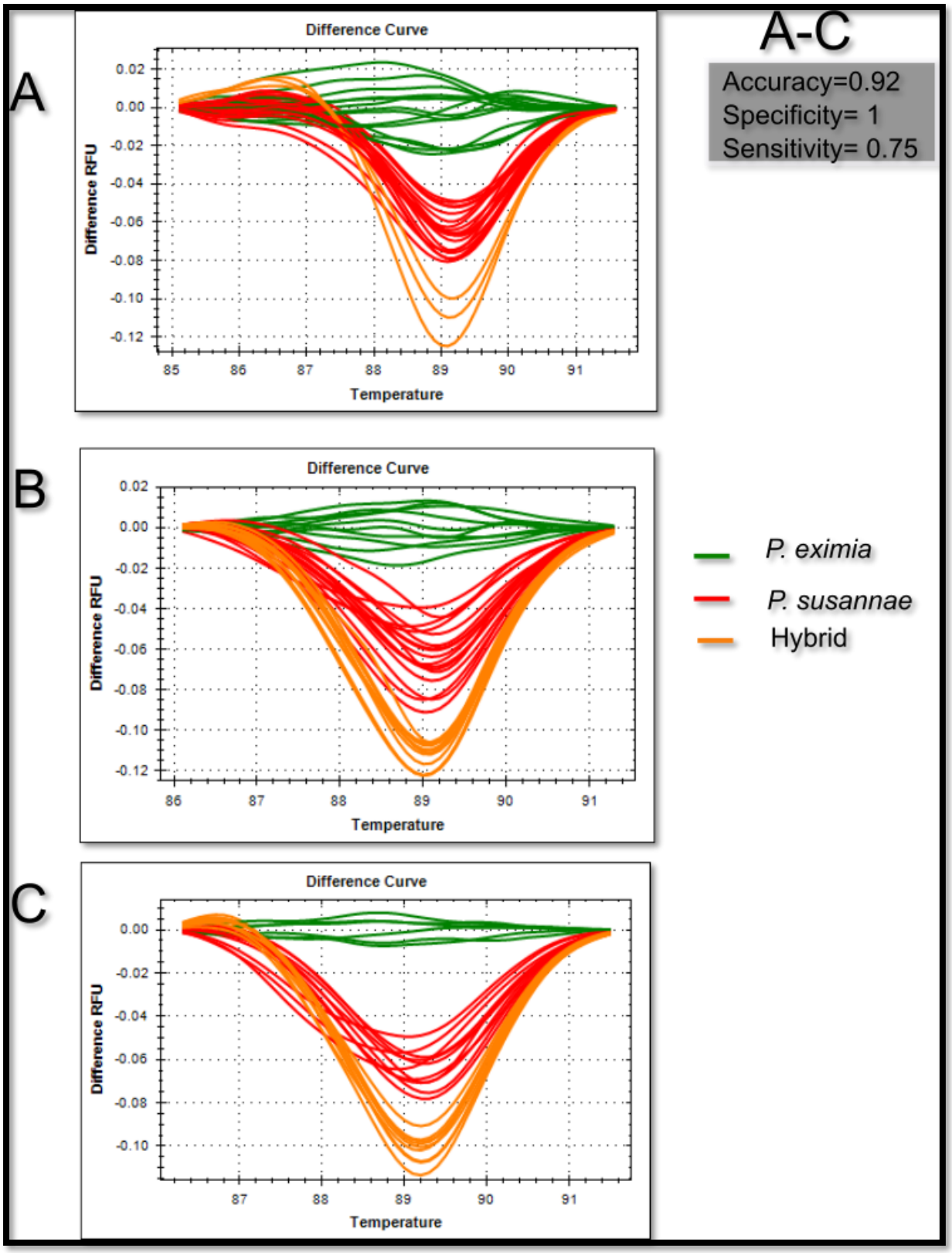


Fig 3.3 A: HRM difference curves for extralimital species and hybrids (A-C)

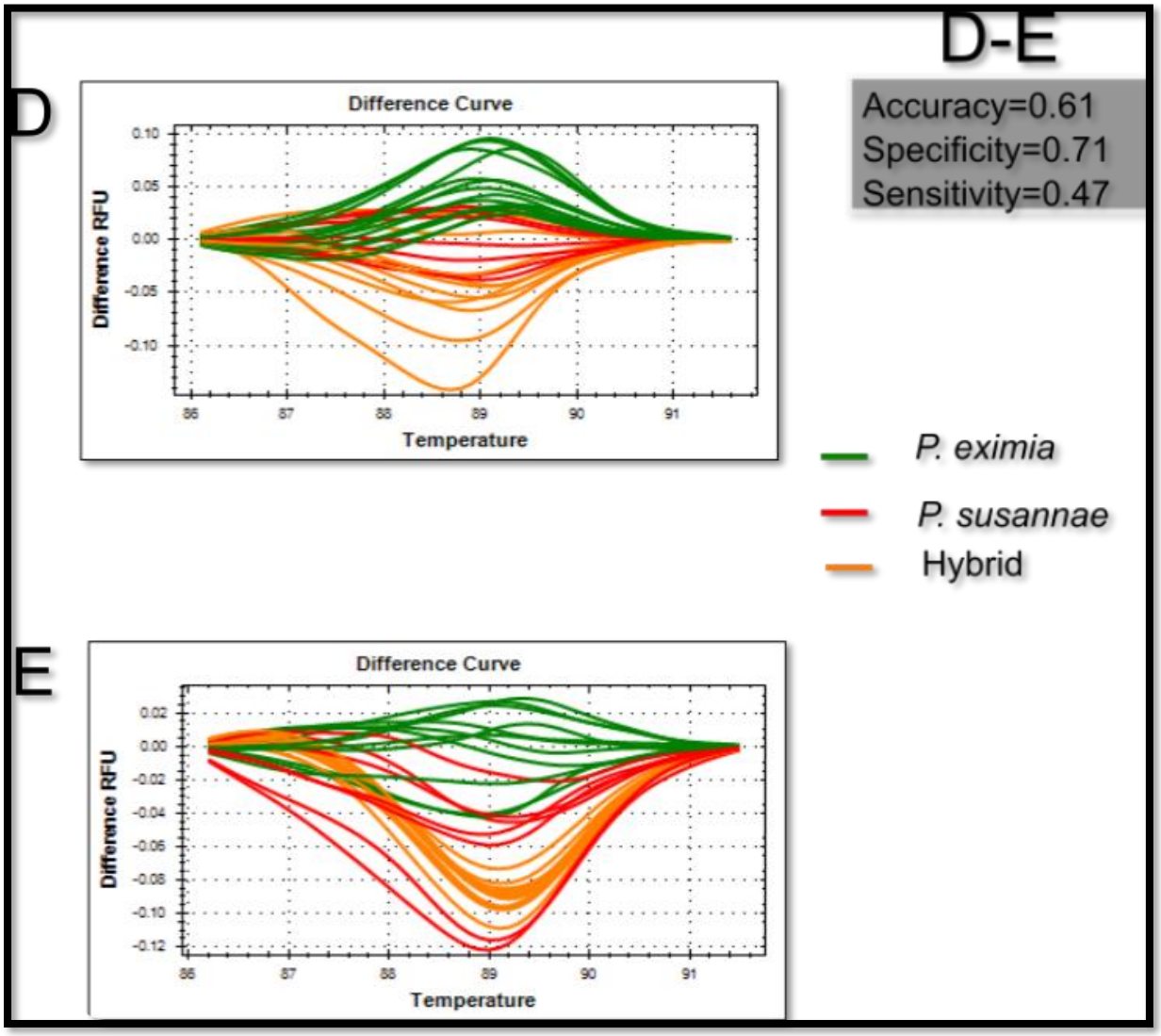


Fig 3.3 B: HRM difference curves for Van Stadens species and hybrids (D-E).

3.3.2 Species-specific primers

The species-specific primers detected more hybrids than Sanger sequencing (Table 3.3). This is likely due to extensive backcrossing occurring between hybrids and the parent species at the Van Stadens Wild Flower reserve as discussed. When backcrossing occurs the proportional number of copies of one species variant may decline thus makes them

difficult to detect as hybrids in the Sanger-sequencing trace files (Table 3.3A). Species-specific primers would detect even lower numbers of species variants. Sanger sequencing suggested that 18 samples were pure *P. eximia*, whereas the species-specific primers detected that 28 samples *were P. eximia* (Table 3.3A; Fig. 3.4). Given that *P. eximia* is the larger population, it is likely that backcrossing of hybrids led to a rapid swamping of the relative proportion of *P. susannae* ITS variants and thus these were not detected in Sanger sequencing trace files.

Table 3.3 A. Comparison of Sanger sequencing results to species-specific primer results for all samples

Sanger sequencing	Species-specific primer: Hybrid	*P. eximia*	*P. susannae*
Hybrid	34	0	0
P. eximia	18	28	0
P. susannae	0	0	18

The species-specific primers only detected 3 *P. susannae* (Table 3.3 B). This was compared to the 18 detected in the larger pool of samples (Table 3.3 A). 34 hybrids were detected by both the sanger sequencing and the species-specific primers over both data sets (Table 3.3 A and B).

Table 3.3 B. Comparison of Sanger sequencing results to species-specific primer results for the Van Stadens Wildflower Reserve samples

Sanger sequencing	Species specific primer: Hybrid	*P. eximia*	*P. susannae*
Hybrid	34	0	0
P. eximia	18	24	0
P. susannae	0	0	3

There was a minor decrease in the frequency of hybrids identified via Sanger sequencing from the pre-fire plants to the post-fire seedlings (Table 3.4). This was due to the 18 samples that were *P. eximia* according to Sanger sequencing, but hybrids according to the species-specific primers (Table 3.3). There was an increase in the frequency of hybrids to pure species from the pre-fire plants to the post-fire seedlings using the species-specific primers This result was shown to be statistically significant (Table 3.4).

Table 3.4. Comparison of hybrid frequency for Van Stadens Wildflower Reserve samples

	Sanger sequencing	Species-specific primers
Pre-fire plants (n=24)	45.8333	45.8333
Post-fire seedlings (n=56)	41.0714	71.4286
P-value from chi square test	0.609	0.018

There were no-*P. susannae* and fewer examples of *P. eximia* picked up by the species-specific primers in the post-fire generation (Fig 3.2;). It appears through this one fire cycle, this system becomes more and more dominated by a hybrid swarm. This is an example of how genetic swamping can take place. This echoes the findings of Laikre et al. (2010): “augmentation through translocation of individuals would lead to a loss of genetic variation, loss of adaptations, change of population composition and change of population structure”. This Van Stadens system is an example of genetic swamping where the local endemic (*P. eximia*) is reduced, the other parent species (*P. susannae*) is not detected and all we are left with is hybrids or variations thereof.

The distribution of plants and their status (pure or hybrid) is shown in Fig. 3.4; the status was determined using the species-specific primers (note that Sanger sequencing and SSP are in agreement for hybrid status reported in Chapter 2). The post fire generation of seedlings consisted of a large proliferation of hybrids distributed evenly across the reserve. It also included three small clumps of *P. eximia* seedlings, two on the north western edge of the reserve and the other on the south eastern corner of the reserve.

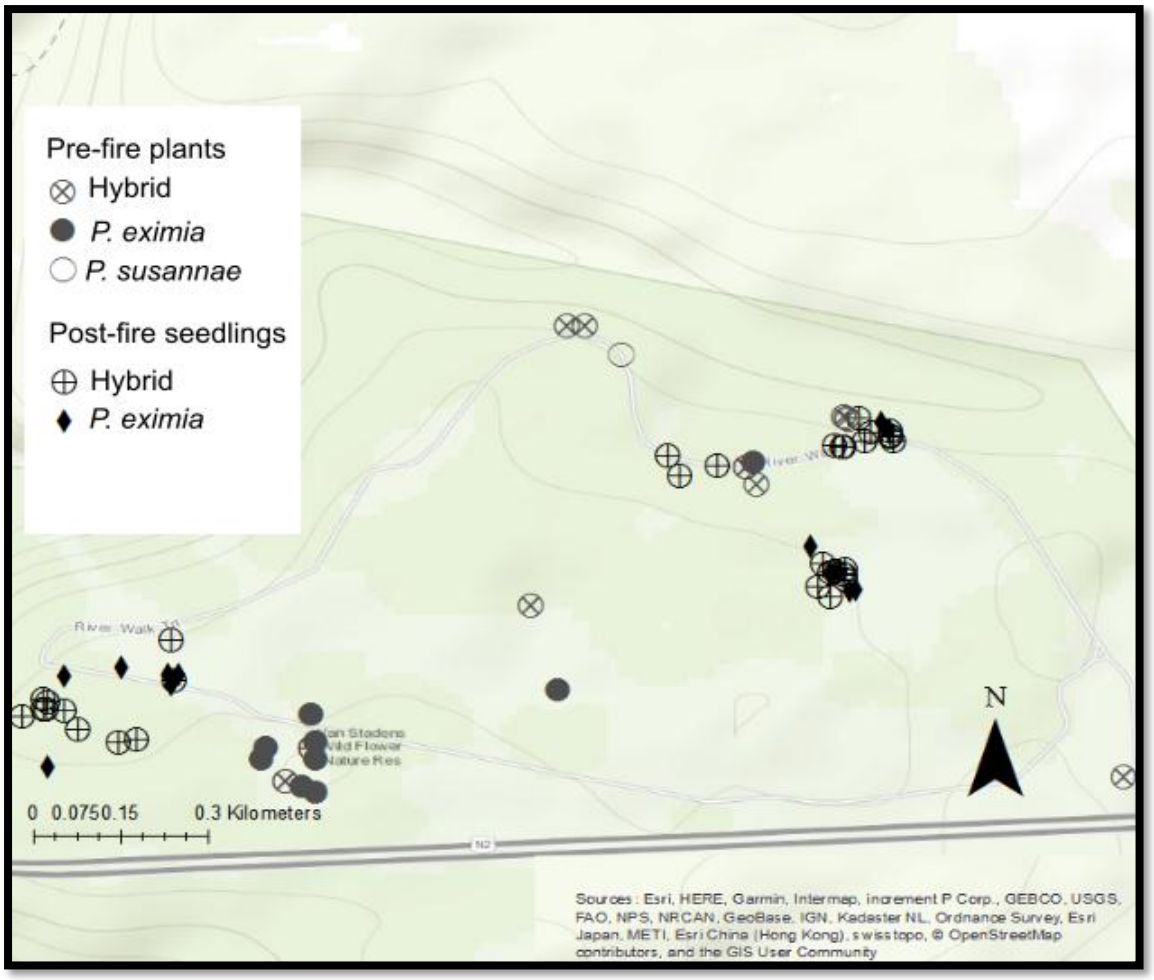


Fig. 3.4. Map of hybrids between *Protea susannae* and *Protea eximia* based on species-specific primer data at Van Stadens Wildflower Reserve

I have shown that the SSP's can provide a new and novel way to genotype or barcode hybrids of *P. eximia* and *P. susannae*. They have shown to be effective. SSP's were shown to be a cheap and successful way to discriminate hybrids from 'pure' species. The SSP's offer a means to detect the spread of hybrids across the landscape. However, SSP's require a fixed species substitution. If this is not detected in the ITS then NGS can be used to screen the genome for it. These SSP's can be adapted for other species when trying to determine the spread of hybridisation between a non-local species and local species that is closely related (Yakandawala and Yakandawala, 2011; Hall, 2016). It is also possible that NGS mapping of samples from a wide geographic range could be done to identify species that can be barcoded with SNP's.

This method of developing species-specific primers (SSP's) is very important for the study of hybridisation. It offers an affordable, high-throughput technique for screening for hybrid

plants. It represents a benchmark which can be added to with further discoveries. These may include calculations of the range, extent and distribution of hybrid plants.

This study also highlighted the role of fire and hybridisation events in Cape fynbos systems. The number of hybrids increased after the fire. It has already been shown that the density of *Protea* shrubs increases after frequent fire events (4-6 years) (Vlok and Yeaton, 2000). However, through this study it is now seen that fire can also increase the number of hybrid offspring and reduce the number of 'pure' parental individuals. This is informative as it means that hybrid zones only may be stable between fire events.

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Chapter 4: Synthesis

Hybridisation has been rarely documented in the Cape and this thesis records the first case of hybridisation due to anthropogenic long distance translocation of plant species within the Cape Floristic Region (CFR). This is a topic that needs to be investigated more widely in the Cape. In addition a new method is developed for its rapid screening cost-effectively, using species-specific primers. However, this only works when the species contain fixed substitutions, i.e. species-specific SNP's in the genome. The use of these methods in other species in the Cape will only be possible if those species and their hybrids contain such markers.

Expanding on the SSP approaches would include perfecting the multiplex PCR methods. In future it would be useful to include the methods to estimate the rate of hybridisation or the rate of spread. An estimated rate of introgression of *P. susannae* into *P. eximia* could help inform conservation efforts in similar Cape systems. Another future expansion of this project could include a survey of the *P. eximia* populations within a 10 km radius (or beyond) of the Van Stadens Wildflower Reserve to determine whether the sugarbirds and *Protea* beetles (*Protea* pollinators) have spread *P. susannae* or hybrid pollen further outside the reserve. For example, we could look to see whether hybridisation has spread to the Lady Slipper Mountain *P. eximia* population. This offers a rare opportunity to calculate a pollen dispersal kernel (Ottewell et al., 2012).

Globally the study of hybridisation has shifted focus to look at how climate change will become a driver of hybridisation (Chunco, 2014; Gómez et al., 2014). Climate change will shift species ranges into new regions and new associations. Species that have never previously come into contact will now meet because of climate change. This thesis offers a glimpse of what these range shifts may cause. It showcases the effects and impacts when species are shifted from one habitat to another.

This study opens additional opportunities for hybridisation studies. There are many future research options available. As discussed in Chapter 3, we can study intergenerational and geographic changes in hybrid frequency from a known ground-zero point of non-local species introduction. Species specific primers (SSP's) of the cpDNA should also be developed to identify maternal parentage. This will be similar to what was done in Chapter 2, but the SSP's will work more rapidly and will be cheaper to produce. Ways need to be found to quantify the proportions of *P. susannae* vs *P. eximia* genes. Also theoretically the SSP's

can be used to run a qPCR to compare relative proportions of parental species (Ginzinger, 2002). Another option would be to investigate hybrid vigour. This would involve determining the rate of survival of the hybrid offspring. Witches broom is a pathogen that predominantly attacks the genus *Protea* (Knox-Davies et al., 1985). It would be interesting to measure the pathogen load of witches' broom on hybrids as compared to pure *P. eximia* and *P. susannae*. This would be to see if the hybrids were inherently weaker than the 'pure' species to the pathogen attack by witches' broom disease (Hooker, 1974).

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Appendix A: Methods in more detail: collection and extraction of DNA (Chapter 2: Materials and Methods)

Plants were semi-randomly collected in the field — we tried to evenly sample from both species. Seven leaves were collected from each shrub. Two leaves of each shrub were placed in paper envelopes and placed in airtight-containers which contained silica gel.

In the lab three 4 mm and one 6 mm glass beads were placed into labelled screwcap tubes along with 0.02 g portion of each silica dried leaf sample. The leaf material was then crushed using a Resch Mixer Mill for 5 min at a frequency of 30 Hz. Eight hundred microlitres of pre-prepared 2X CTAB (per sample) was placed in a falcon tube and 1 µl of mercaptoethanol was added to the falcon tube per sample under a fume hood. The solution was heated to 65 °C in a water bath on a heating block. Eight hundred micro-litres (800 µl) of the solution in the falcon tube was added to each sample. The samples were inverted for 5 min. All samples were incubated at 65 °C and 1040 rpm for 1 h. Then the samples were removed from the incubator. Three hundred micro-litres (300 µl) of chloroform:isoamyl alcohol (24:1) and 300 µl 90% ethanol were added to each sample. They were then centrifuged at 12,500 rpm for 15 min (Sahu et al., 2012). Five hundred to 550 µl supernatant of each sample was pipetted into an uncontaminated 1.5 ml reaction tube. Then 300 µl ice cold isopropanol, 300 µl 99% ethanol and 36 µl 5M Ammonium acetate solution were added to each sample (Prunier and Latimer, 2010). To precipitate the DNA, the samples were placed in a −20 °C freezer for a minimum of an hour. The samples were centrifuged at 12,000 rpm for 15 min to recover the DNA. The supernatant was tipped out into a waste bottle so only the DNA pellet remained. The DNA pellet was further washed out with 250 µl of 75% ethanol, while the

DNA pellet was agitated from the side of the tube with a clean pipette tip while doing this. Samples were then centrifuged at 12,000 rpm for 15 min and ethanol tipped out into a waste bottle. Opened tubes were placed in an airtight container filled with silica crystals for 2 h for the ethanol to evaporate. DNA was suspended in 50 µl of molecular water and placed in the fridge for an hour. DNA quality and quantity were then tested on a ThermoScientific Nanodrop 2000c Spectrophotometer.

A master mix containing Ampliquon AccuPOL™ DNA Polymerase Ready Mix, molecular grade water, and dilute ITS4 and 5m primers was produced. The master mix was vortexed for 2 min. The quantities of each component added differed per the number of samples tested. These quantities were calculated on an MS Excel Spreadsheet. The 34.8 µl master mix was added to 1.2 µl, 10^{-1} dilution, of each sample of DNA in 0.2 ml PCR reaction tubes. The PCR cycling conditions were template denaturation at 80.8 °C for 5 min followed by 30 cycles of denaturation at 95.8 °C for 1 min, primer annealing at 50.8 °C for 1 min, followed by a ramp of 0.38 °C/s to 65.8 °C, and primer extension at 65.8 °C for 4 min; followed by a final extension step of 5 min at 65.8 °C as per Shaw et al. (2014). To detect whether PCR amplification was successful the PCR product was checked using electrophoresis of an agar gel at 100 V for 10 min. Successful PCR amplicons were sequenced by Inqaba Biotec™ (Pretoria). Sequences were then contigged and aligned using Codon Code Aligner version 6.0.2 (Centerville, Massachusetts).

The same steps were used to obtain the cpDNA sequences using trnV(UAC)x2 and ndhC primer pair (Shaw et al., 2014).

Appendix B: Sequence chromatograms (Chapter 2: Introduction)

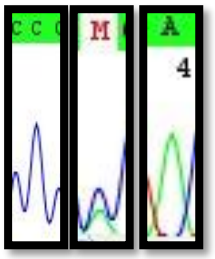

Figure B1: Sequence chromatograms for C (*P. eximia*), M (hybrid) and A (*P. susannae*).

Appendix C: HRM cleaning procedure (Chapter 3 Materials and Methods)

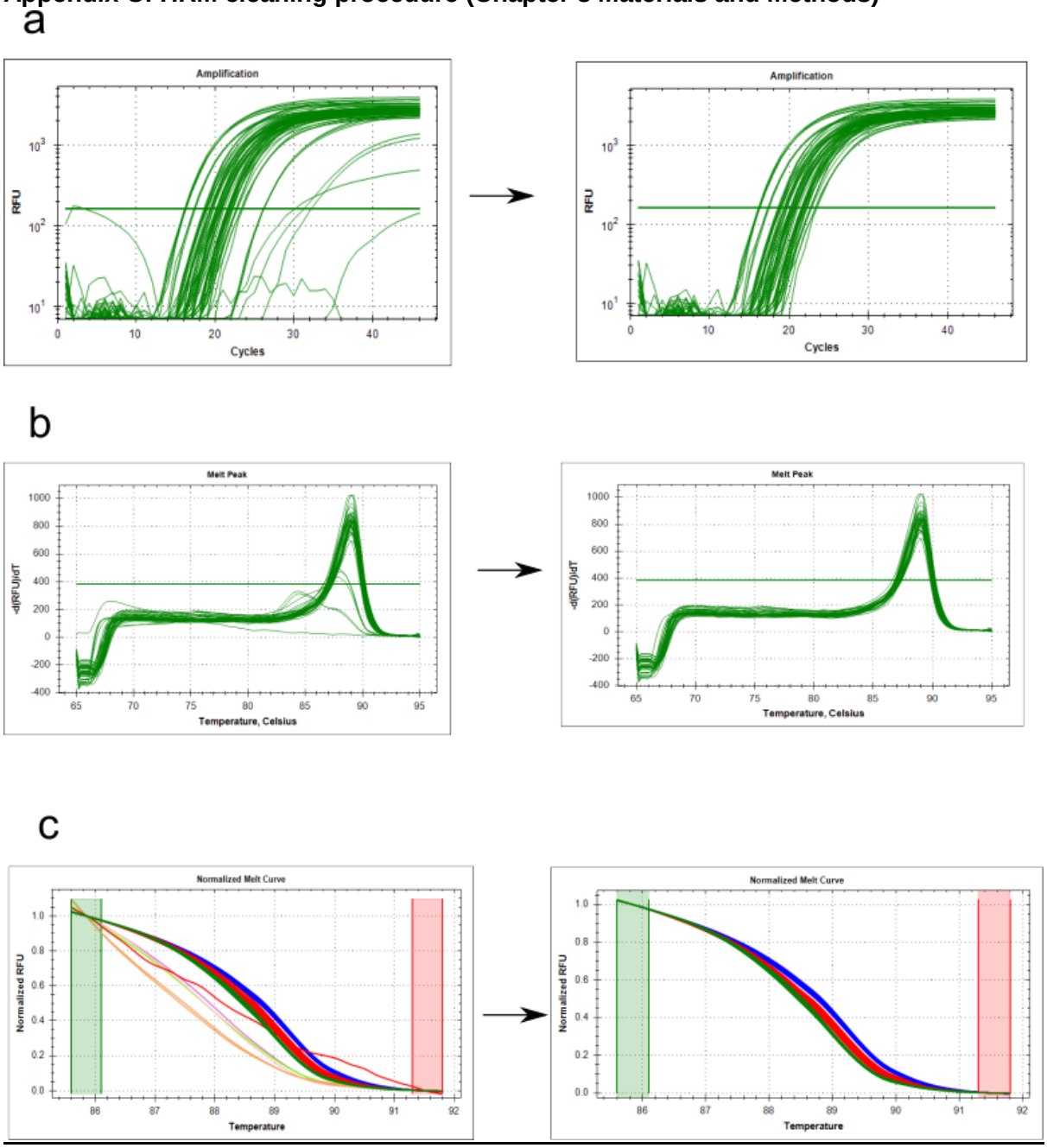

Fig. C1: Advanced cleaning procedure to eliminate any PCR's that may have failed. a: Log graph cleaning, b: Melt Peak cleaning and c: Normalized Melt Curve cleaning.

Appendix D: R script (Chapter 3: Materials and Methods)

```
---
  title: "IntoTheBreach"
author: "Alastair Potts & Nicholas Galuszynski"
date: "05 October 2017"
modified: "31 October 2018"
output: html_document
---
install.packages('reshape2',repos='http://cran.us.r-project.org')
install.packages('tidyr',repos='http://cran.us.r-project.org')
setwd("C:/Users/Timothy/Dropbox/Protea R")
dat <- read.csv("PROTEA_HRM.csv")
senseless.function <- function(primer) {
  dat  %>% group_by(Gene,Cluster) %>% count() %>% tidyr::spread(Cluster,n) %>%
as.data.frame -> res
  rownames(res) <- res$Haplotype
  res <- res[,-1]
  res[is.na(res)]<-0

  RES2 <- NULL
  for (i in 1:nrow(res)) {
    maxcell.col <- which(res[i,]==max(res[i,]))[1]
    truepositive <- res[i,maxcell.col]
    falsenegative <- sum(res[i,])-truepositive
    falsepositive <- sum(res[,maxcell.col])-truepositive
    truenegative <- sum(res)-truepositive-falsenegative-falsepositive
    n <- sum(res)
```

```
    sensitivity <- truepositive/(truepositive+falsenegative)

    specificity <- truenegative/(truenegative+falsepositive)

    accuracy <-
(truepositive+truenegative)/(truepositive+falsepositive+truenegative+falsenegative)

    RES2 <-
rbind(RES2,c(n,truepositive,truenegative,falsepositive,falsenegative,sensitivity,specificity,
accuracy))

  }

  colnames(RES2) <-
c("n","truepositive","truenegative","falsepositive","falsenegative","sensitivity","specificity","acc
uracy" )

  rownames(RES2) <- c("Hybrid", "P.exim", "P.sue")

return(RES2)

}
```

Appendix E: Primer names and sequences (Chapter 3: Materials and Methods)

Table E1: Names of the primers created and their respective sequences

Primer name	Sequence 5'→ 3'
5'FwdA_19	ACGCGTACGTCGCCCGCCA
5'FwdC_19	ACGCGTACGTCGCCCGCCC
5'FwdA_18	CGCGTACGTCGCCCGCCA
5'FwdC_18	CGCGTACGTCGCCCGCCC
5'RevA_19	CGCCACAGGGACGGCAGGT
5'RevC_19	CGCCACAGGGACGGCAGGG
5'RevC_18	GCCACAGGGACGGCAGGG

Appendix F: Cost Analysis

Table F1: Cost analysis of Sanger sequencing

Item	Cost individually		No. of samples	Final Cost
Blue tips 1000µl (1000 bag)	R 0.21	per tip	50	R 10.40
Yellow tips 200µl (1000 bag)	R 0.17	per tip	50	R 8.25
White tips 10µl (1000 bag)	R 0.13	per tip	50	R 6.65
1.5ml epi microcentrifuge reaction tubes	R 0.29	per tube	50	R 14.40
Thin wall flat cap clear PCR tubes	R 0.34	per tube	50	R 17.05
Molecular grade water (1L)	R 0.26	per ml	50	R 13.20
Agarose	R 4.67	per mg	50	R 233.50
Latex Gloves N/powder	R 0.69	per glove	50	R 34.40
Isopropanol 500ml	R 0.52	per ml	50	R 26.00
Ethanol (analyzed analytical reagent) 500ml	R 0.58	per ml	50	R 29.18
Chloroform (99.8%) 1L	R 1.23	per ml	50	R 61.40
EDTA	R 1.97	per g	50	R 98.68
Tris base 1kg	R 3.78	per g	50	R 188.92
Sequencing	R 90.10		50	R 4 505.00
			No. reagents used	
Ampliquon TAQ DNA POL 2X MMIX RED 1.5 ML 100RXN	R 330.60		2	R 661.20
Pronosafe nucleic acid stain (CK130-CONDA)	R 4.92	per reaction	50	R 246.00
Total cost				R 6 154.23

Table F2: Cost analysis of species-specific primers

Item	Cost individually		No. of samples	Final Cost
Blue tips 1000µl (1000 bag)	R 0.21	per tip	50	R 10.40
Yellow tips 200µl (1000 bag)	R 0.17	per tip	50	R 8.25
White tips 10µl (1000 bag)	R 0.13	per tip	50	R 6.65
1.5ml epi microcentrifuge reaction tubes	R 0.29	per tube	50	R 14.40
Thin wall flat cap clear PCR tubes	R 0.34	per tube	50	R 17.05
Molecular grade water 51200 1l	R 0.26	per ml	50	R 13.20
Agarose	R 4.67	per mg	50	R 233.50
Latex Gloves N/powder	R 0.69	per glove	50	R 34.40
			No. reagents used	
Ampliquon TAQ DNA POL 2X MMIX RED 1.5 ML 100RXN	R 330.60		2	R 661.20
Pronosafe nucleic acid stain (CK130-CONDA)	R 4.92	Per reaction	50	R 246.00
Total cost	R 342.28			R 1 245.05

Table F3: Cost analysis of HRM

Item	Cost individually		No. of samples	Final Cost
Blue tips 1000µl (1000 bag)	R 0.21	per tip	50	R 10.40
Yellow tips 200µl (1000 bag)	R 0.17	per tip	50	R 8.25
White tips 10µl (1000 bag)	R 0.13	per tip	50	R 6.65
1.5ml epi microcentrifuge reaction tubes	R 0.29	per tube	50	R 14.40
Molecular grade water 51200 1l	R 0.26	per tube	50	R 12.94
Latex Gloves N/powder	R 0.69	per ml	50	R 33.71
			No. used	
Hard shell 96 Well plates (50)	R 73.68		2	R 147.36
Precision supermix for HRM	R 23.62	Per reaction	50	R 1 181.00
Total Cost	R 99.05			R 1 414.71

Hybrid meme